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In this report, we describe our progress during the past year (6/15/95-6/15/96) in studying different aspects of the signaling pathways initiated by the EGF receptor and the related Neu/ErbB2 tyrosine kinase. We have obtained evidence for a novel mechanism for growth factor signaling where growth factor-stimulated dimerization events (e.g. between the EGF receptor and Neu or between Neu and ErbB3) are transient and give rise to secondary receptor dimers. We also have obtained increasing evidence for the importance of two new signaling molecules, the c-Cbl protein and an 18 kDa nuclear GTP-binding protein (p18) in the actions of the EGF receptor and Neu.

In the coming year, we intend to extend these studies and identify the specific cellular proteins that are activated in human breast cancer cells following primary and secondary receptor dimerization events. We also will determine whether the Cbl proto-oncogene product serves to interface the EGF receptor with the Cdc42 GTP-binding protein in breast cancer cells and gives rise to a signaling pathway that culminates in the activation of p18. Finally, we will determine if identical signaling pathways are operating in dog mammary carcinomas as an important step toward validating this system as a model for human breast cancer.

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## INTRODUCTION

The overall goal of the work outlined in the original proposal was to further our understanding of the signal transduction pathways initiated by members of the EGF receptor subfamily of receptor tyrosine kinases, with a particular emphasis on Neu/ErbB2. The reason for this was that a number of lines of evidence seemed to implicate Neu in the development of human breast cancer. Within this section of the annual report (for the period of July 15, 1995 to July 15, 1996), I will present the background of the research problem (including some updates of the research developments from our group and other laboratories over the past year), a review of the purpose of the present research, and the methods of approach that are being used.

**1. Background of the Research Problem**- Growth factor receptor tyrosine kinases (1-4) have attracted much interest because of their importance in initiating signaling pathways that are critical for normal cell growth and because of their relevance to understanding the biochemistry of human cancer. The epidermal growth factor (EGF) receptor subfamily, which has been the focus of our studies, includes the EGF receptor, the Neu tyrosine kinase (also often referred to as ErbB2 or HER2) (5,6), the ErbB3 (HER3) protein (7,8), and ErbB4 (HER4) (9). Each of these receptors appears to be comprised of a single polypeptide chain ($M_r$ ~170-180 kDa), consisting of a cysteine-rich extracellular (growth factor-binding) domain, a single membrane-spanning helix, and a cytoplasmic tyrosine kinase domain. As alluded to above, a variety of lines of evidence have implicated the EGF receptor-subfamily in different types of cancer. The amplification of each of the 4 members of the EGF receptor family has been reported in different mammary carcinoma cells; Neu/ErbB2, in particular, has been found in a significant proportion of human breast cancers (up to 40%) and appears to have a significant predictive value for the overall survival of patients with breast cancer (10-16). Because of these correlations, a major focus of our original proposal was to understand the molecular regulation of the Neu/ErbB2 tyrosine kinase and the signaling implications of the activation of its tyrosine kinase activity.

It has generally been agreed that the growth factor-stimulated dimerization of receptor tyrosine kinases is an essential aspect of their regulation and function (17). Much of the importance of receptor dimer formation may lie in the intermolecular (trans) phosphorylation events which enable the binding of specific cellular phospho-substrates and the initiation of multiple signaling pathways. These trans-phosphorylation events are not only an important outcome of receptor homodimer formation (e.g. EGF receptor--EGF receptor interactions) but also occur following the formation of different receptor heterodimers. This has important implications regarding the activation of Neu/ErbB2 because it has been well documented that the EGF receptor will form a heterodimer with Neu in an EGF-dependent manner (18-21). Moreover, we and others (22,23) have shown that Neu tyrosine kinase activity is stimulated by the growth factor, heregulin [also known as Neu-differentiation factor (NDF)], as an outcome of heregulin-promoted heterodimer formation between ErbB3 (or ErbB4) and Neu.

Intense efforts to identify growth factors for Neu led to the discovery of NDF, a 44 kDa glycoprotein that was first isolated from Ras-transformed fibroblasts (24-26), and the family of heregulins, which represent the human homologs of NDF (27). However, not all cells that express Neu/ErbB2 respond to heregulin (28), which led to the findings that the actions of the heregulins (and NDF) are mediated through ErbB3 or ErbB4. Thus, receptor heterodimer formation provides a mechanism by which a receptor tyrosine kinase (e.g. Neu) can be activated by a specific ligand (heregulin) that it normally does not bind. This also provides the possibility for an individual growth factor (EGF, heregulin) to activate different combinations of receptors and thereby increase the diversity of signaling pathways that it can initiate.

Recently, we have found that the situation is more complicated than originally anticipated (Friedberg et al., manuscript in preparation-also see "Progress Report"). We have found that the addition of either EGF or heregulin to cells can give rise to a series of receptor heterodimers. For example, EGF addition first stimulates EGF receptor--Neu interactions, but following trans-phosphorylation, Neu dissociates from this primary dimer and forms a secondary dimer with ErbB3. Heregulin addition first stimulates ErbB3--Neu heterodimer formation but these

heterodimers then give rise to secondary dimers formed between Neu and the EGF receptor. Thus, as will be elaborated upon in more detail in the "Progress Report", we now suspect that growth factor signaling occurs as a series of signaling 'waves', with each wave being triggered by the formation of a specific receptor heterodimer.

Certainly, an important step following the formation of a receptor heterodimer and the ensuing receptor trans-phosphorylation events is the recruitment of cellular proteins (in most cases through the binding of SH2 domain-containing proteins to the tyrosine phosphorylated receptors) which represent the individual building-blocks for signal transduction pathways. A number of lines of evidence have led to the delineation of a signal pathway that includes the activated Ras GTP-binding protein and which appears to connect growth factor binding at the cell surface to the stimulation of MAP kinase activity (i.e. the Erks) in the nucleus (29-31). However, there are a number of other proteins that also bind directly to (phosphorylated) growth factor receptors and it now seems likely that the responses from multiple signaling pathways need to be integrated in order to achieve proper regulation of cell growth. During the past year, we have found an interesting example of how EGF binding to the membranes of human breast cancer cells leads to the specific phosphorylation of the Cbl proto-oncogene product. At present, the role that the ~125 kDa Cbl protein plays in growth factor-coupled signal transduction is unknown. However, there are a number of reasons to suspect that Cbl may function as an important cell growth regulator, since various studies have very recently shown that Cbl is a major phospho-substrate for tyrosine kinases in hematopoietic cells (32-34) and that its homolog in *C. elegans*, the *sli-1* gene product, serves to antagonize the actions of the *let-23* gene product (i.e. the EGF receptor homolog) in vulval development (35). An important step toward delineating the role of Cbl in EGF signaling in breast cancer cells will be to determine its downstream signaling partners and recently we have shown that one such partner may be the Crk proto-oncogene product (see below).

In addition to identifying the cytosolic proteins that mediate growth factor receptor signaling to the nucleus, another important challenge is to identify the nuclear proteins that receive these signals and directly translate them into effects on cell-cycle regulation. We have identified a ubiquitous 18 kDa GTP-binding protein (designated p18) which may play such a role. The GTP-binding activity is activated by EGF and heregulin as well as by exposure of cells to UV radiation. The latter finding suggests that p18 may play a role in a stress-response pathway similar to the nuclear MAP kinases, the c-Jun kinase (JNK1) and p38 (36,37). Thus, an important future research direction will be to determine the growth factor-coupled signaling pathway that leads to the activation of p18.

**2. Purpose of the Present Research/Experimental Methods**- The research goals for the work being supported by the Department of Defense in the coming year will continue to focus on understanding the molecular basis by which the Neu tyrosine kinase and other members of the EGF receptor family stimulate signaling pathways that influence cell growth, and under some conditions contribute to the development of human breast cancer. Our original working hypothesis for the stimulation of Neu tyrosine kinase activity by heregulin was that heregulin binds to its true receptor, ErbB3 (or ErbB4), and promotes the formation of a heregulin--ErbB3--Neu complex. Within this complex, heregulin could directly contact Neu and induce the stimulation of its tyrosine kinase activity. The activated Neu could then trans-phosphorylate ErbB3 (or ErbB4), which would serve to recruit other cellular signaling molecules that are capable of binding to the tyrosine-phosphorylated receptors (e.g. via the SH2 domains of the signaling proteins). These interactions would constitute the first step in the construction of signaling pathways that emanate from the receptor tyrosine kinases and presumably lead to the nucleus. While we have obtained data that is consistent with the formation of heregulin-stimulated Neu--ErbB3 heterodimers in various cells, our work over the past year has pointed to a new but more complicated mechanism for growth factor activation. We now are thinking of growth factor-stimulated signaling as a series of receptor heterodimerization events, where one ligand (e.g. heregulin) induced receptor heterodimerization event gives rise to the trans-phosphorylation of receptors and the formation of secondary (ligand-independent) dimers.

During the past year, we also have identified a predominant cytosolic tyrosine phospho-substrate for the EGF receptor in human breast cancer cells (i.e. the Cbl proto-oncogene product) and a nuclear GTP-binding protein (p18) whose activity is regulated by both EGF and heregulin and which may play a role in cell cycle-check point control. In the coming year, we plan to examine each of these areas of growth factor signaling, namely the primary and secondary receptor dimerization events that occur in the plasma membrane, the role of the Cbl protein in EGF signaling through the cytosol, and the mechanisms of regulation of the nuclear p18 protein. As we characterize each of these signaling activities from the plasma membrane to the nucleus, we also will continue to compare the results we obtain in human breast cancer cells with the results we obtain in dog mammary carcinoma cells, in order to validate the dog system as a possible model for experimental therapeutic intervention against breast cancer. Overall, we feel that we are exactly on schedule in terms of the kinds of studies that we originally anticipated for year 3 of the grant.

In performing these studies, we will use a combination of biochemistry and recombinant DNA technology. The studies on receptor heterodimer formation will involve the use of monoclonal antibodies that are specific for the EGF receptor, Neu, ErbB3, and ErbB4 in order to selectively precipitate these individual receptors from cells and then determine what other receptor-partners are co-precipitated. We also will use site-directed mutagenesis and cDNA transfection approaches to introduce different mutated Neu molecules into cells as a means of identifying the critical tyrosine residues that need to be phosphorylated in order for Neu to dissociate from a primary receptor heterodimer and then to bind in a ligand-independent manner to other receptors (ErbB3, the EGF receptor) to form secondary receptor heterodimers. In the studies aimed at further characterizing EGF signaling through the Cbl proto-oncogene product, we will use specific antibodies raised against Cbl to precipitate the protein and determine under what conditions other cellular proteins bind to Cbl. We also are developing baculovirus expression systems for full length Cbl and different truncated versions of the Cbl protein as glutathione-S-transferase (GST) fusion proteins in *Spodoptera frugiperda* (insect cells). Our intention is to use the GST-Cbl proteins to probe lysates from human breast cancer cells for potential signaling partners. This will involve first stimulating the cells with EGF and then exposing the lysates to GST-Cbl for varying periods of time, followed by precipitation of the GST-Cbl with glutathione-agarose and then examining the re-suspended precipitates by SDS-PAGE to detect associated cellular proteins. For our studies of the nuclear GTP-binding protein that we suspect plays a role in heregulin- and/or EGF-mediated cell-cycle check-point control, we will use purification approaches to isolate sufficient amounts of the protein for micro-sequencing analysis. This will represent the first step toward the molecular cloning of the cDNA for p18. We also will use cDNA transfection approaches to determine whether p18 is acting downstream from the Ras-like GTP-binding protein Cdc42 and from the nuclear MAP kinases, JNK1 and p38.

## BODY/"PROGRESS REPORT"

The following is a progress report regarding the work that has been performed by our laboratory during the past year (6/15/95-6/15/96) as funded by the Department of Defense (DOD) Breast Cancer Initiative USAMRDC grant DAMD17-94-J-4123. This report is subdivided into the following sections, 1.) Description of objectives, 2.) Progress of the research, 3.) Manuscripts resulting from the research (indicated in bold, below), 4.) Personnel involved in the studies, and 5.) Meetings where the research will be presented.

**1. Description of Objectives**- The original proposal that we submitted to the DOD was centered on our finding that the growth factor heregulin, which was initially thought to bind directly to the Neu/ErbB2 tyrosine kinase, in fact bound to a related member of the EGF receptor subfamily, ErbB3 [Carraway et al., (1994) J. Biol. Chem. 269, 14303-24306]. Independently, another research group found that heregulin can also bind to ErbB4 [Plowman et al. (1993) Nature 366, 473-475]. These findings led us to hypothesize that the ability of heregulin to stimulate the tyrosine kinase activity of Neu/ErbB2 in human breast cancer cell lines was due to a heregulin-

stimulated heterodimer between ErbB3 (and/or ErbB4) and Neu. We proposed that heregulin binding to ErbB3 would promote the formation of a ternary heregulin--ErbB3--Neu complex, resulting in the (heregulin-stimulated) activation of Neu and the trans-phosphorylation of ErbB3. Our expectation was that the trans-phosphorylated ErbB3 would recruit cellular proteins that contained Src-homology 2 (SH-2) domains, including c-Src, which would initiate signaling pathways that influenced cell-cycle progression and cell growth. Thus, our major aims were to determine that heregulin-stimulated ErbB3--Neu interactions occurred in cells, as indicated by co-precipitation of these receptors using specific anti-ErbB3 and anti-Neu antibodies, and that these interactions led to the tyrosine phosphorylation of ErbB3 (*in vivo*). We also wanted to determine whether other combinations of the EGF receptor subfamily (e.g. the EGF receptor and Neu or the EGF receptor and ErbB3) formed heterodimers in cells. The formation of different receptor heterodimers would raise the possibility that EGF as well as heregulin could activate Neu in breast cancer cells. Our longer range goals were to determine what other signaling proteins might be phosphorylated and/or activated by these receptor tyrosine kinases in human breast cancer cells and whether similar kinds of receptor--signaling protein interactions and phosphorylation events occurred in dog mammary cancer cells. Such findings would be an important first step in establishing the suitability of spontaneous mammary carcinomas that occur in the dog as a model for human breast cancer.

During the first year of support, we made substantial progress toward achieving many of the objectives that were outlined in the original proposal. We demonstrated that heregulin-stimulated interactions between ErbB3 and Neu occurred in cells and that these interactions led to the phosphorylation of ErbB3 and the formation of a complex between phosphorylated ErbB3 and the 85 kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase. We also demonstrated that other receptor heterodimers were capable of forming in cells, including EGF receptor--Neu dimers and EGF receptor--ErbB3 dimers. In addition, we identified two potentially new signaling molecules, a 130 kDa protein (designated p130) whose phosphorylation was strongly stimulated by EGF and an 18 kDa GTP-binding protein (designated p18) whose activity was stimulated by both EGF and heregulin.

Based on these findings, we proposed the following five aims for the second year of support: 1.) to determine whether heregulin-stimulated ErbB3--p85 interactions occurred in mammary carcinoma cells, 2.) to determine the mechanisms underlying the activation of Neu/ErbB2 by the EGF receptor, 3.) to further characterize the interaction between the EGF receptor and p130, 4.) to further characterize the 18 kDa GTP-binding protein, and 5.) to begin to assess the suitability of dog mammary carcinoma cells as a model system for human breast cancer.

In the past year, we have been able to pursue each of these lines of investigation and in many cases, this work has led to interesting new findings. We have examined the ability of heregulin to stimulate ErbB3--p85 interactions in more detail and have found that like the case for rat pheochromocytoma (PC12) cells (which we have used as a model system to study different members of the EGF receptor subfamily), ErbB3--p85 interactions also occur in a heregulin-stimulated manner in human breast cancer cells. Through these studies, and other work aimed at understanding how EGF stimulates the activation of Neu/ErbB2, we made the novel finding that receptor dimer formation is a transient process (i.e. analogous to enzyme--substrate interactions) and that primary receptor dimer formation gives rise to new sets of secondary receptor dimers. This had led us to propose a new model for growth factor receptor signaling, where the signaling events occur in 'waves', with the first signaling wave originating from the primary receptor dimer and the second signaling wave then occurring as an outcome of secondary dimer formation. We also have determined that the 130 kDa phospho-substrate for the EGF receptor which we discovered in the first year of our studies is the Cbl proto-oncogene product. We have found that this protein is specifically activated as an outcome of EGF receptor homo-dimer formation and that a consequence of this activation in human breast cancer cells is the formation of a complex between Cbl and the Crk proto-oncogene product. In addition, we have obtained a significant amount of new information regarding the 18 kDa GTP-binding protein (p18) whose activity is stimulated by EGF and heregulin, including the finding that p18 couples to a cell-cycle-check point control protein called RCC1. We also have been examining possible signaling molecules that act upstream

of p18, in particular the Ras-like GTP-binding protein Cdc42, and this has led us to make some interesting and novel findings regarding the activation of Cdc42 and related GTP-binding proteins by a family of oncoproteins and by the lipid phosphotidylinositol 4,5 bisphosphate. Finally, we have begun to assess whether similar kinds of phosphorylation events occur in dog mammary cancer cells as observed in human breast cancer cells. Using the Cbl protein as an indicator, we have been able to show that this protein is highly phosphorylated in dog mammary cancer cells that express high levels of EGF receptor, exactly as is the case for human breast cancer cells. All of these findings will be summarized in more detail below.

## 2. Progress of the Research:

**2A. Discovery of a novel mechanism for growth factor-coupled signaling-** In previous studies funded by the DOD, our laboratory demonstrated that heregulin promotes the formation of receptor heterodimers between the Neu tyrosine kinase and ErbB3 and that this resulted in the tyrosine phosphorylation of ErbB3 [Gamett et al. (1995) J. Biol. Chem. 270, 19022-19027]. When we extended these initial observations, we made the surprising finding that the tyrosine phosphorylated ErbB3 protein (but not Neu) forms a tight complex with the 85 kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase. This then suggested that the heregulin-stimulated Neu--ErbB3 heterodimer represented a transient complex rather than a stable complex, such that following the trans-phosphorylation of ErbB3, it dissociates from Neu and is able to bind to cellular target/effector molecules. Thus, ErbB3 appeared to be acting as a signaling transducer, somewhat reminiscent of G proteins in hormone receptor signaling pathways, where the G protein initially forms a complex with the hormone-bound receptor, but after the receptor-catalyzed activation of the G protein, it dissociates from the receptor and forms a new complex with an effector/enzyme.

During the past year, we have discovered that the mechanisms underlying growth factor-stimulated heterodimer formation are more complicated than the relatively straightforward model outlined above, but nonetheless pose some novel and interesting implications regarding growth factor-coupled signaling. This discovery, which has formed the basis of a manuscript soon to be submitted (**Friedberg et al.**), began with the finding that treatment of PC12 cells with EGF resulted in the precipitation of ErbB3 when using a specific anti-Neu antibody (Figure 1). This was similar to what we had observed following treatment of the cells with heregulin; however, the important difference was that EGF does not bind to either Neu or ErbB3. Thus, the Neu--ErbB3 complex that formed in response to EGF treatment must be the outcome of a secondary receptor dimer. The idea is that EGF first stimulates the formation of an EGF receptor--Neu heterodimer but after EGF-stimulated trans-phosphorylation of Neu, it dissociates from the EGF receptor and forms a new dimer with ErbB3.

In a separate set of experiments, we found that when PC12 cells were first stimulated by heregulin, and then Neu was immunoprecipitated with a specific anti-Neu monoclonal antibody and the tyrosine kinase activity in the resuspended pellet was assayed in the presence of the tyrphostins AG879 (a specific Neu inhibitor) and AG556 (a specific EGF receptor inhibitor) (38), the tyrosine kinase activity was not only inhibited by AG879 but also by AG556. This suggested that another protein tyrosine kinase (e.g. the EGF receptor) co-precipitated with Neu. We then verified this through Western blot analyses, where we examined anti-Neu immunoprecipitates for the presence of the EGF receptor. We found that when the experiment was performed in the presence of the EGF receptor-specific tyrphostin, AG1478, a strong EGF receptor band was found in the Neu immunoprecipitates. On the other hand, we have found that inhibition of Neu tyrosine kinase activity by the specific Neu antagonist AG879, significantly reduced the presence of the EGF receptor in Neu immunoprecipitates.

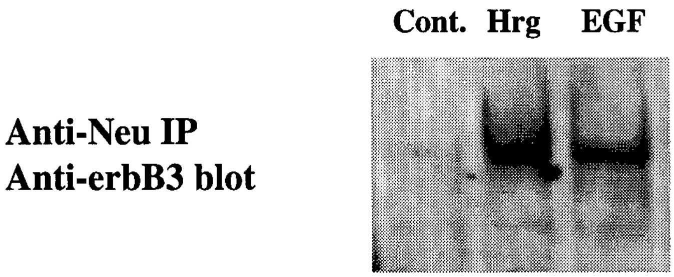


Figure 1

These results appear to be consistent with the following scheme presented in Figure 2. The addition of heregulin (HRG) to cells stimulates the formation of receptor heterodimers between Neu and either ErbB3 (as shown in Figure 2) or Neu and ErbB4. In the former case, heregulin

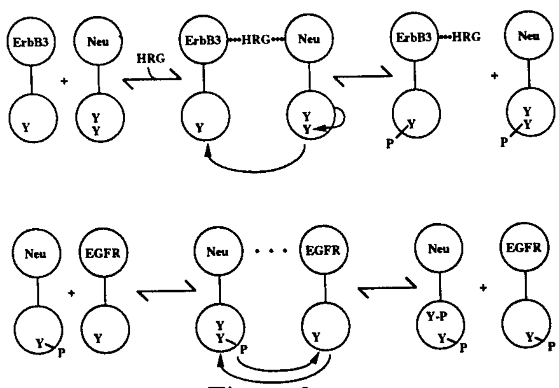


Figure 2

binding to Neu within the ternary complex stimulates Neu tyrosine kinase activity leading to increased autophosphorylation and trans-phosphorylation of ErbB3. Since ErbB3 shows no detectable tyrosine kinase activity, there is no trans-phosphorylation (by ErbB3) of Neu. The phosphorylation of ErbB3 then results in its dissociation from Neu, such that ErbB3 is free to associate with p85 as we initially observed [Gamett et al. (1995) J. Biol. Chem. 270, 19022-19027]. However, the autophosphorylated Neu is now activated and able to bind to other (non-phosphorylated, inactive) receptors, in particular the EGF receptor. This then results in the formation of a secondary (ligand-independent) dimer between Neu and the EGF receptor and further trans-phosphorylation events. The trans-phosphorylation of Neu by the EGF receptor would presumably result in the dissociation of this secondary dimer, such that Neu and the EGF receptor would then be free to interact with specific cellular phospho-substrates. Thus, we find that the inhibition of EGF receptor tyrosine kinase activity stabilizes the formation of the secondary dimer and thereby increases the amount of EGF receptor detected in Neu immunoprecipitates. However, the inhibition of Neu tyrosine kinase activity would prevent the dissociation of the (heregulin-stimulated) primary dimer between Neu and ErbB3 (or ErbB4) and thereby inhibit secondary dimer formation.

Overall, these findings now suggest that growth factor-stimulated dimer formation between members of the EGF receptor family is analogous to a typical enzyme--substrate interaction, where one monomeric receptor tyrosine kinase acts as an enzyme, catalyzing the trans-phosphorylation of the partner monomeric receptor (the substrate). Once the receptor serving as a substrate is phosphorylated, it becomes the product of the enzyme reaction and dissociates from the receptor kinase (i.e. the enzyme). Such a mechanism raises a number of important implications. First, the trans-phosphorylation of a receptor such as Neu within the primary dimer represents an activation

event, such that Neu is able to seek out other receptors and phosphorylate them in a ligand-independent manner. This then would explain earlier findings made by our laboratory that EGF addition to cells appears to result in the activation of Neu tyrosine kinase activity (i.e. as an outcome of EGF-stimulated EGF receptor--Neu primary dimer formation). Secondly, this mechanism would now suggest that growth factor-coupled signaling in fact occurs as 'waves of signaling events'. The first wave would represent the interactions of cellular phospho-substrates with monomeric, phosphorylated receptors that arise from the primary receptor dimerization event, and then a second wave of signaling events would follow secondary dimer formation. Because Neu-ErbB3 heterodimers can either form as primary dimers (in a heregulin-dependent fashion) or as secondary dimers (following EGF-stimulated EGF receptor--Neu primary dimer formation and potentially following heregulin-stimulated ErbB4--Neu primary dimer formation), it will be important to determine which cellular phospho-substrates bind to phosphorylated ErbB3 for each of these different cases. For example, does p85 complex with ErbB3 as an outcome of the heregulin-stimulated Neu--ErbB3 primary dimer or following the formation of a secondary dimer between Neu and ErbB3? It also will be important to determine if Neu is essential for primary and secondary dimer formation (i.e. is it a unique property of Neu that allows these events to occur) and do human breast cancer cells and dog breast cancer cells show similar dimerization events as originally detected in PC12 cells?

**2B. Identification of a specific EGF-stimulated phospho-substrate in human breast cancer cells as the Cbl proto-oncogene product**- During the past year, we set out to identify cellular (cytosolic) proteins whose phosphorylation was strongly stimulated by EGF or by heregulin in human breast cancer cells. While performing such a screen in MDA-MB-468 human mammary carcinoma cell lysates, we found a 130 kDa protein (originally designated p130) that became highly tyrosine phosphorylated in an EGF-dependent manner and bound to GST-Src-SH3 and GST-Src-SH2 fusion proteins (Figure 3, below; also see **Flanders et al., submitted**). We subsequently found that p130 also bound effectively to the amino-terminal SH2 domain of the adaptor molecule, Grb-2. In order to determine the identity of p130, we probed blots with antibodies against several 120-130 kDa proteins. Two 130 kDa proteins that were of

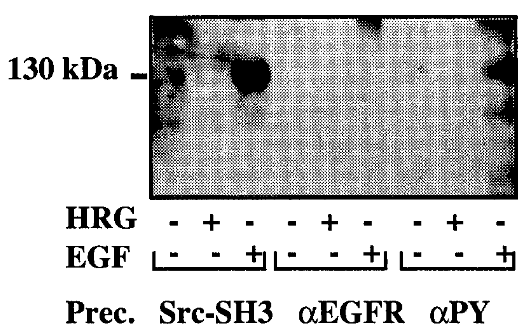


Figure 3

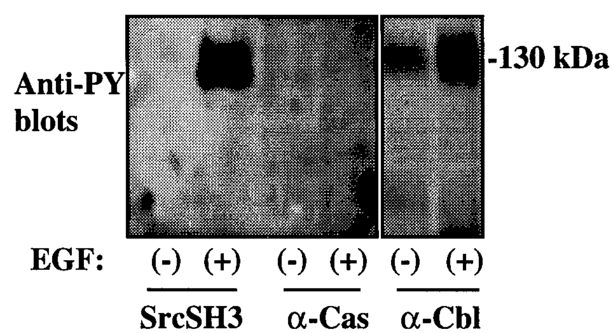


Figure 4

particular interest were c-Cbl and c-Cas (39,40); c-Cbl was of interest because it has been found to be heavily tyrosine phosphorylated in hematopoietic cells in response to cytokines while Cas was of interest because it is a major phospho-substrate for the Src tyrosine kinase. We found that both c-Cbl and c-Cas could be precipitated from MDA-MB-468 cell lysates with both GST-Src-SH2 and GST-Src-SH3 fusion proteins; however, only the c-Cbl protein bound to GST-Grb2-SH3 and was tyrosine phosphorylated after EGF stimulation (see Figure 4 which shows that when Cbl was immunoprecipitated with a specific anti-Cbl antibody it was phosphorylated on tyrosine residues in an EGF-dependent manner). In addition, unlike Cas, c-Cbl was not detected to co-

immunoprecipitate with anti-Src antibody. Thus, we have concluded that p130 is the Cbl proto-oncogene product.

We have found that EGF stimulates the tyrosine phosphorylation of c-Cbl in another breast cancer cell line, SKBR3, which over-expresses the EGF receptor, Neu and ErbB3. However, MDA-MB-453 human mammary carcinoma cells, which do not over-express the EGF receptor, do not show an EGF-stimulated tyrosine phosphorylation of c-Cbl. We also have found that heregulin does not stimulate the tyrosine phosphorylation of Cbl in any human mammary carcinoma cell that we have thus far examined (despite the fact that Neu and ErbB3 are over-expressed in some of these cells). Taken together, these results indicate that the tyrosine phosphorylation of Cbl is a specific outcome of EGF receptor activation and occurs in breast cancer cells where the EGF receptor is over-expressed.

The specific mechanism underlying the coupling of Cbl to the activated EGF receptor in breast cancer cells is still not known, although it is probably mediated at least in part by the binding of Cbl to the Grb-2 adaptor molecule. The amino-terminal SH3 domain of Grb-2 associates with Cbl, while the SH2 domain of Grb-2 binds to a phospho-tyrosine residue within the carboxyl terminus of the EGF receptor. Thus, it seems likely that EGF-stimulated activation of the EGF receptor can recruit a Grb-2--Cbl complex, resulting in the phosphorylation of Cbl.

An important question concerns the nature of the signaling pathway that is initiated by the EGF-stimulated tyrosine phosphorylation of Cbl in human breast cancer cells. Because during our initial investigations of p130 we suspected that this protein might be the c-Src substrate, Cas, and because Cas has been shown to form a complex with the Crk proto-oncogene product, we examined whether p130 might be capable of binding to Crk. We found that this in fact was the case, with an anti-Crk antibody co-precipitating the Crk and Cbl proteins in an EGF-dependent manner (**see Figure 5 in Flanders et al.**). The Crk proto-oncogene product is a 40 kDa cytoplasmic adapter protein which contains an amino terminal SH2 domain followed by two SH3 domains (41,42). Our preliminary evidence indicates that the SH2 domain of Crk binds to the tyrosine phosphorylated Cbl protein. However, it is interesting that we find that the EGF-dependent Cbl--Crk interaction in human breast cancer cells might be mediated by a prior association of c-Crk with the EGF receptor. To our knowledge, there have been no previous reports of *in vivo* binding of c-Crk to the EGF receptor. It is possible that the EGF receptor--Crk association is a specific characteristic of the MD-MB-468 breast cancer cells, since neither constitutive binding of c-Crk to the EGF receptor, nor EGF-stimulated tyrosine phosphorylation of c-Crk was detected in human epidermoid carcinoma (A431) cells. Thus, if simply high levels of EGF receptor were necessary to provide detectable EGF receptor--Crk complexes, we would have expected little difference between the MD-MB-468 cells and A431 cells. However, it is possible that the levels of expression of Crk are high in the breast cancer cells and thus give rise to a constitutive association between Crk and the EGF receptor.

We find that the time course for the EGF-stimulated phosphorylation of Cbl appears to correlate with the interaction between Cbl and c-Crk. Taken together, our data are consistent with a scheme where the phosphorylation of Cbl results in its uncoupling from the EGF receptor and its association with Crk, which in turn leads to the formation of a distinct Crk--Cbl complex. This is somewhat reminiscent of what we originally observed for ErbB3 and the 85 kDa regulatory subunit of the phosphatidylinositol 3-kinase in work supported by the DOD [Gamett et al., (1995) J. Biol. Chem. 270, 19022-19027]. In that case, heregulin first stimulated the formation of a complex between Neu and ErbB3 and the resulting trans-phosphorylation of ErbB3 led to its dissociation from Neu and promoted the formation of a new complex between ErbB3 and p85.

A key question for the future concerns the signaling implications for the formation of an EGF-stimulated Cbl--Crk complex in human breast cancer cells. One possibility is that Cbl acts to interface Crk and Crk-binding proteins (e.g. the Abl tyrosine kinase) with the EGF receptor in mammalian epithelial cells. However, it also is possible that Cbl plays some type of negative-regulatory role in the actions of the EGF receptor, based on the findings that the homolog for c-Cbl in *C. elegans*, the Sli-1 protein, has been reported to antagonize EGF-stimulated signaling (35). For example, EGF-stimulated phosphorylation of Cbl may enable it to interact with other signaling molecules that normally bind to the EGF receptor (i.e. EGF-stimulated phosphorylation of Cbl

may allow it to interact with Crk and prevent Crk from binding to its normal signaling partners). Thus, an important aim for the future will be to examine these possibilities further as well as to determine the identity of the cellular signaling proteins that associate with Cbl and Crk in human breast cancer cells.

**2C. Cbl is a specific EGF receptor phospho-substrate in dog mammary carcinoma cells-** Given that the EGF-stimulated tyrosine phosphorylation of the c-Cbl protein, and its subsequent association with c-Crk, were specific and readily identifiable features of human breast cancer cells that highly express the EGF receptor, we have used these read-outs to compare the similarities between human and dog mammary carcinomas. We find that the dog mammary carcinoma cell line (CMT12) which over-expresses the EGF receptor yields a strongly (EGF-stimulated) phosphorylated protein at ~130 kDa with essentially identical mobility to p130 in human breast cancer cells (MD-MB-468) and in human A431 cells. We also have found that the 130 kDa tyrosine phosphorylated protein, when immunoprecipitated from CMT12 cells with an

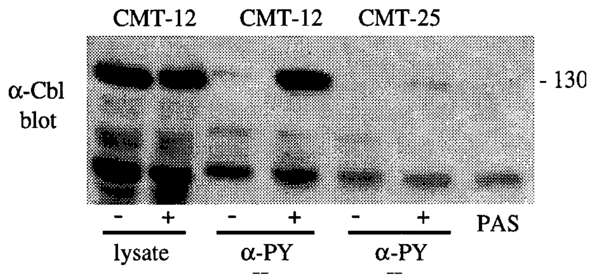


Figure 5

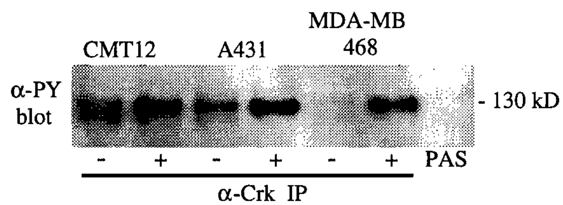


Figure 6

anti-phosphotyrosine antibody, can be Western blotted with an anti-Cbl antibody (Figure 5; PAS represents a control lane where protein-A Sepharose was used alone). However, another dog mammary carcinoma cell line, CMT25, which does not over-express the EGF receptor does not show an EGF-stimulated tyrosine phosphorylation of the c-Cbl protein, just as is the case for the human MD-MB-453 cells which do not express the EGF receptor. We also have found that c-Crk is tyrosine phosphorylated in an EGF-dependent manner in dog mammary carcinomas and that an anti-Crk antibody will co-precipitate Crk and Cbl in an EGF-dependent manner, just as is observed in human A431 cells and in human breast cancer (MDA-MB-468) cells (Figure 6). Based on these preliminary results, we are encouraged that the signaling pathways that are operating in dog mammary carcinomas that over-express the EGF receptor are similar to those operating in human breast cancer cells.

**2D. Identification and biochemical characterization of a novel growth factor-stimulated nuclear GTP-binding protein-** The original goal of these studies was to extend growth factor signaling pathways to the nucleus and to determine whether GTP-binding proteins might act as molecular switches to mediate signaling by the EGF receptor or Neu or related growth factor receptors. To this end, we have identified a nuclear, 18 kDa GTP-binding activity, designated p18, which is highly sensitive to the addition of growth factors to $G_0$ arrested cells **(Wilson et al., submitted)**. In PC12 cells, we found that this activity is most strongly stimulated by nerve growth factor (NGF), followed by heregulin and then EGF, whereas in HeLa cells, the activity is stimulated most effectively by heregulin (**Figure 1A in Wilson et al.**). While much of our work has taken advantage of the sensitivity of PC12 cells to NGF and heregulin (i.e. stimulated neurite extension), we have found p18 in every cell line which we have investigated (including all human breast cancer cells that have been examined). The ubiquitous nature of this protein suggests that it may be playing a fundamental role in growth factor signaling at the level of the nucleus.

It is interesting that although p18 responds immediately to growth factor addition, its GTP-binding activity does not become maximal until after ~1 hour of growth factor treatment, indicating that the GTP-binding activity is not subject to down-regulation in this time frame. We also have found that p18 is able to bind GTP when cells are arrested in the G1/S phase of the cell cycle, but not in $G_0$ or M phase. Thus, it is possible that growth factor activation of p18 is necessary to initiate the cell to exit $G_0$ and then once activated, p18 must retain its activity to allow progression of the cell cycle.

A potentially important biochemical characteristic of p18 activity is that it is activated within a larger protein complex, which is eluted through gel filtration columns at an apparent mass of 150 kDa. Fractions containing monomeric p18 (i.e. at 18 kDa) do not show GTP-binding activity, unless p18 is pre-labeled with [$\alpha^{32}$P]GTP prior to chromatography. This suggests to us that a regulator of the GDP-GTP exchange activity of p18 is present in the larger molecular mass complex (of 150 kDa) and that once p18 binds GTP, it can then dissociate from this complex. An important question concerns the identity of the p18 regulator. RCC1 was an obvious candidate since it has already been shown to catalyze the guanine nucleotide exchange reaction (43) of Ran, a 25 kDa nuclear GTP-binding protein, and because a 17 kDa nuclear GTP-binding protein has been shown to associate with the *Saccharomyces cerevisiae* RCC1 homolog, PRP20 (44). Using the temperature-sensitive cell line, tsBN2, to investigate whether RCC1 could act as a stimulator of the guanine nucleotide exchange activity of p18, we found that the loss of RCC1 did influence the GTP-binding activity of p18. However, instead of causing a concomitant decrease in GTP-binding activity as would be expected if RCC1 were an activator of p18, we instead found that the loss of RCC1 caused a striking increase in the ability of p18 to bind GTP (**see Figure 6A in Wilson et al.**). In addition, we found that p18 GTP-binding activity could be detected in the cytosol when RCC1 levels were decreased. These observations, taken together with the finding that p18 co-immunoprecipitates with RCC1 (**see Figure 6B in Wilson et al.**), are consistent with a direct interaction between p18 and RCC1. One provocative possibility is that RCC1 is acting as a GDP-dissociation-inhibitor (GDI) for p18, such that when it is no longer available to bind p18, the p18 protein is then free to be activated by a guanine nucleotide exchange factor. This change in the interplay between a GDI and a guanine nucleotide exchange factor may be the outcome of a specific growth factor-coupled signaling pathway and could provide the mechanistic basis by which growth factor addition to cells leads to stimulated GTP-binding by p18.

**2E. Possible relationship between growth factor signaling through Rho-like GTP-binding proteins (Cdc42/Rac) and activation of p18**- A key question for the future concerns the specific signaling pathway that gives rise to the activation of p18. While one documented pathway from the plasma membrane to the nucleus involves the Ras--Raf--Mek--Erk signaling cascade, our preliminary results suggest that the inhibition of Ras signaling (by use of a dominant-negative Ras mutant in PC12 cells) does not affect the ability of p18 to respond to growth factor treatment. Another interesting possibility involves stress-activated signaling pathways, since we have found that p18 responds to UV-light exposure as well as to growth factor treatment (K. Wilson, unpublished data). Of particular interest is the pathway that begins with the activation of the PAKs (p21-activated serine /threonine kinases) by Cdc42 or Rac and results in the stimulation of the stress-activated nuclear MAP kinases JNK1 and p38 (45-47). A number of lines of evidence show that signaling pathways initiated by Cdc42 and related GTP-binding proteins (Rac, RhoA) are activated by growth factors and oncogenes. Recently, we have shown that both the Cdc42 and RhoA GTP-binding proteins are activated by a potent oncogene product designated Dbl (because this oncogene was first found in DNA from Diffuse B cell lymphomas). The transforming activity of Dbl both required its ability to bind and activate the GTP-binding proteins and to be targeted to a cytoskeletal location within the cell (**Zheng et al., 1996; in press**). We have been able to show that activation of the GTP-binding proteins is mediated by a distinct domain of ~200 amino acids on the Dbl oncoprotein, designated the DH (Dbl-homology) domain which is found in a number of other oncoproteins, and that targeting Dbl to a specific cytoskeletal ligand is mediated by the Pleckstrin-homology (PH) domain of the Dbl molecule. It now appears that in many cases, Cdc42 and related GTP-binding proteins will be activated by members of the

Dbl family. However, in other cases, it appears that members of the Dbl family will need to work together with other factors to activate the GTP-binding proteins and initiate their signaling pathways. One possible cofactor is phosphatidylinositol 4,5 bisphosphate (PIP2). Recently, we have shown that PIP2 will bind to the carboxyl terminal domain of the Cdc42 GTP-binding protein, as well as to the Rac and RhoA GTP-binding proteins, and will strongly stimulate GDP dissociation from these proteins (**Zheng et al., submitted**). Growth factor-stimulated hydrolysis of PIP2 (i.e. through the actions of phospholipase C-$\gamma$) may reduce the levels of this lipid sufficiently to allow cellular GTP to bind to the nucleotide-depleted Cdc42 and result in its activation. However, it also is possible that PIP2 will work together with some Dbl-related proteins to promote the activation of Cdc42 or Rac or RhoA. In the future, we hope to obtain information regarding what factors may work together with the family of Dbl oncoproteins to stimulate signaling pathways initiated by Cdc42 and if this in turn results in the activation of p18. We also will be interested in determining whether the expression of a constitutively active PAK or JKN1 promotes p18 activation. In addition, it will be of interest to determine if there is some connection between the signaling pathway initiated by Cbl (see Section 2B, above) and those initiated by Dbl-like proteins, since all of these proteins have been suggested to be cytoskeletal-associated.

## CONCLUSIONS

**1. Significance of the Research-** The signal transduction pathways that are initiated by growth factor receptor tyrosine kinases (particularly members of the EGF receptor family) have been the central focus of the studies funded by DOD because it is clear that these signaling systems have a major impact on the development of human cancers. This is especially true for members of the EGF receptor subfamily, and in particular for Neu/ErbB2, since these receptors appear to be highly (aberrantly) expressed in human breast cancers and there appears to be a strong correlation between the expression of Neu and the prognosis for the breast cancer patient (10-16).

During the past year, we have achieved a good deal of what we outlined as goals in our previous "Progress Report" and have obtained some interesting and important new insights into the signaling systems that underlie cell growth regulation. We have uncovered a rather novel mechanism for growth factor-promoted receptor dimer formation, where it now appears that the initial binding of EGF to the EGF receptor, or the initial binding of heregulin to ErbB3 (or ErbB4), triggers a primary receptor dimerization event [i.e. between the EGF receptor and Neu or between Neu and ErbB3 (or ErbB4), respectively]. However, following receptor trans-phosphorylation, the primary receptor dimer dissociates into its component monomers and these (phosphorylated) monomeric receptors can then initiate secondary receptor dimerization events. This cascade of receptor dimerization events has some important implications regarding how growth factor-initiated signaling occurs, since it suggests that signaling really represents a defined series of events for each dimerization.

We also have been able to identify one of the predominant EGF-stimulated tyrosine phospho-substrates in human breast cancer cells as the product of the Cbl proto-oncogene. At present little is known about the Cbl proteins and its involvement in signal transduction, although previous work has shown that amino-terminal truncated forms of Cbl are strongly transforming and that a homolog of Cbl antagonizes signaling in *C. elegans* that is essential for vulval formation. We also have found that Cbl binds to the product of another proto-oncogene, i.e. the c-Crk protein, in an EGF-dependent manner. Thus, the Cbl and Crk proteins may participate in an important new signaling pathway in breast cancer cells that is initiated by the EGF receptor.

Finally, we have continued our work on a novel nuclear GTP-binding protein, p18, whose activity is strongly stimulated by heregulin in a variety of cells including human breast cancer cells. We have determined that p18 is stimulated during G1/S of the cell cycle and that it appears to associate with the DNA-binding protein, RCC1. We now suspect that the p18-RCC1 interaction is involved in maintaining p18 in the nucleus and in a GDP-bound state, until a growth factor signal releases p18 and thereby enables it to undergo GDP-GTP exchange. We also feel that p18 is likely

to be downstream from the Cdc42 (or Rac) GTP-binding proteins and thus we have begun to study the activation of this pathway by an interesting family of oncoproteins. Overall, our work has highlighted a number of novel participants (e.g. Cbl, p18, Dbl-related proteins) in growth factor-stimulated signaling pathways. We intend to determine if these participants also play important roles in growth factor-signaling in dog mammary cells and hope to use this information to design new strategies for therapeutic intervention against breast cancer that might ultimately be testable in a dog model.

**2. Methods of Approach: Plans for the Coming Year**- During the next year, we will expand on each of the lines of study outlined in the "Progress Report". We will continue our work on the formation of primary and secondary receptor heterodimers and set out to determine their implications on growth factor-coupled signaling. In particular, we will establish if distinct groups of cellular proteins are phosphorylated and/or activated as an outcome of each receptor dimerization event. We will extend our work with the p130/Cbl protein that we have recently identified in human breast cancer cells and establish the signaling consequences of EGF-stimulated Cbl--Crk interactions. We also will further delineate the signaling pathways that are initiated by heregulin interactions with ErbB3 and Neu and culminate in the activation of the nuclear GTP-binding protein, p18. An important goal of these studies will be to determine how growth factor receptor tyrosine kinases communicate with the GTP-binding protein, Cdc42, since this may be an important upstream event for the eventual activation of p18. Finally, we will continue to assess whether each of the protein--protein (signaling) interactions detected in human breast cancer cells also occur in a similar manner in dog mammary carcinomas, in order to ascertain the validity of the dog model for designing therapeutic strategies against human breast cancer. These various research goals underlie the following specific experimental aims.

**i.) Role of primary and secondary receptor dimer formation in growth factor signaling in human breast cancer cells**- Much of our work showing the formation of primary and secondary receptor heterodimers has been performed in rat PC12 cells, which is a model system that we have used to study the actions of Neu/ErbB2 because it gives rise to a readily assayable biological response (i.e. neurite extension). For example, we have found that following EGF addition to PC12 cells, a primary heterodimer can form between the EGF receptor and Neu. Following the trans-phosphorylation of Neu, it dissociates from the EGF receptor and forms a secondary dimer with ErbB3. We also have shown that following the formation of a heregulin-stimulated heterodimer between Neu and ErbB3, a secondary dimer forms between Neu and the EGF receptor. We will determine the mechanistic basis for these dimerization events and identify the tyrosine phosphorylation site(s) on Neu that causes it to initially dissociate from the EGF receptor (i.e. for the case of an EGF receptor--Neu primary dimer) or from ErbB3 (for the case of a Neu--ErbB3 primary dimer) and then in a ligand-independent manner to form secondary dimers (with ErbB3 or the EGF receptor, respectively). In the coming year, we also will establish just how general a phenomena this is by determining whether primary and secondary receptor heterodimers also form in breast cancer cells and in lung cancer cells and if certain receptor combinations are favored in certain cancer cells. Finally, through careful time-course studies and immunoprecipitation experiments, we will set out to identify the specific cellular proteins that are phosphorylated as an outcome of a particular primary receptor dimer (e.g. EGF receptor--Neu) or through the formation of a specific secondary receptor dimer (Neu--ErbB3).

**ii.) Biochemical studies of the Cbl proto-oncogene product and its involvement in signaling in human breast cancer cells**- We recently have shown that EGF addition to human breast cancer cells results in the tyrosine phosphorylation of the c-Cbl protein and its association with c-Crk (via its SH2 domain). During the course of these studies, we found that Crk appears to constitutively associate with the EGF receptor in certain human breast cancer cells (MD-MB-468 cells) and that the EGF stimulated phosphorylation of Cbl appears to be correlated with a dissociation of Crk from the EGF receptor. In the coming year, we will determine the nature of the constitutive association between Crk and the EGF receptor (i.e. if it occurs via the

SH2 domain of Crk and tyrosine-phosphorylated EGF receptors that are generated in the absence of EGF), since this protein--protein interaction may be unique to breast cancer cells that highly express both proteins. We also will establish exactly how Cbl binds to the EGF receptor and if following EGF-stimulated phosphorylation, Cbl binds to Crk in a manner that causes the Cbl--Crk complex to dissociate from the receptor. Finally, an important goal of this series of studies will be to identify other proteins that participate in the signaling pathway initiated by the Cbl--Crk interaction. Since Cbl has been reported to be associated with the cytoskeleton, we will determine whether Cbl binds directly to F-actin and/or to other signaling molecules that have been implicated in mediating EGF-promoted cytoskeletal changes. Of particular interest is the GTP-binding protein Cdc42. We suspect that Cdc42 initiates a signaling cascade that culminates in the activation of the nuclear MAP kinases JNK1 and p38, as well as in the activation of a novel nuclear GTP-binding protein, p18 (see below). At present, the specific mechanism by which the EGF receptor inputs into the Cdc42 signaling cascade is unknown, although an attractive possibility that we will examine in detail is whether the EGF-stimulated phosphorylation of Cbl provides a possible interface between this receptor tyrosine kinase and Cdc42. One way in which we will examine this possibility will be to immunoprecipitate Cbl from breast cancer cells and then determine whether Cdc42 or Cdc42-regulatory proteins (i.e. Dbl-like proteins) are present in a complex with Cbl. Another possibility that will receive serious consideration is that Cbl--Crk interactions result in the activation of the Abl tyrosine kinase. The loss of regulation of Abl already has been implicated in human leukemias, but little is known regarding its involvement in other human cancers. Because Abl is able to associate with Crk, it will be interesting to determine whether the EGF-stimulated formation of a Cbl--Crk complex ultimately gives rise to a change in Abl tyrosine kinase activity in human breast cancer cells.

**iii.) Biochemical characterization of the nuclear GTP-binding protein, p18**- During the next year, we will continue our studies on p18 with the intention of determining the participants of the EGF- and heregulin-stimulated signaling pathways that lead to p18 activation. We will establish whether dominant-negative mutants of Cdc42, that bind to Dbl regulatory proteins and block the activation of endogenous Cdc42 molecules, inhibit growth factor stimulation of p18 activity. If we find this to be the case, we then will set out to determine how different members of the EGF receptor family are able to mediate the activation of Cdc42 through the stimulation of Dbl-related proteins. As described in the preceding section, one possibility that we will examine is that Dbl proteins that contain SH3 domains bind to a proline rich region on the c-Cbl protein. We also will follow-up on our recent findings that p18 appears to bind to RCC1, a nuclear protein that has been implicated in chromosomal condensation and cell-cycle-check point control, and that p18 association with RCC1 both maintains the nuclear localization of p18 and keeps p18 in an inactive state. If this is true, then growth factor signaling through the Cdc42-PAK-JNK (or p38) pathway may give rise to the dissociation of p18 from RCC1, perhaps as an outcome of a phosphorylation of RCC1 by PAK or JNK (or p38). We will examine this possible regulatory mechanism as well as explore the possibility that a glutathione-S-transferase (GST)-RCC1 fusion protein, which we have generated in the laboratory, might be used as an affinity step to isolate purified preparations of p18 for microsequence analysis as a first step toward the molecular cloning of this protein.

**iv.) Continued assessment of dog mammary carcinomas as a model for human breast cancer**- Thus far, we have found that dog mammary carcinomas behave like human breast cancer cells with regard to the EGF-stimulated tyrosine phosphorylation of c-Cbl. As we continue to elucidate the individual steps in the signaling pathways that are responsible for EGF-stimulated tyrosine phosphorylation of c-Cbl (and Cbl--Crk interactions) and the EGF- and heregulin-promoted activation of p18, we will determine whether these same protein--protein interactions can be detected in dog mammary carcinomas. If we are able to verify that very similar signaling pathways are operating in dog mammary cells, we then will begin to determine whether inhibitors that are generated for these pathways (i.e. kinase-defective mutants of Neu, phosphorylation-defective mutants of ErbB3 or Cbl, guanine nucleotide exchange-defective

mutants of Cdc42) show similar effects on the growth of human breast cancer cells and dog mammary carcinoma cells.

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## MANUSCRIPTS RESULTING FROM THE RESEARCH FOR THE PERIOD 6/15/95-6/15/96-

1. Flanders, J.A., Thompson, A.M., Knudsen, B., and Cerione, R.A. Identification of c-Cbl as a signaling partner for the EGF receptor and c-Crk in mammary cancer cells. Submitted to *J. Biol. Chem.*
2. Wilson, K.F., Singh, U.S., Lannigan, D., Macara, I., and Cerione, R.A. Identification of a growth factor-stimulated GTP-binding activity in the nucleus. Submitted to *J. Biol. Chem.*
3. Zheng, Y., Zangrilli, D., Cerione, R.A., and Eva, A. The Pleckstrin homology domain mediates transformation by oncogenic Dbl through Specific Intracellular Targeting. *J. Biol. Chem.* in press.
4. Zheng, Y., Wu, W.J., and Cerione, R.A. Phosphatidylinositol 4,5 bisphosphate (PIP2) provides an alternative to guanine nucleotide exchange factors by stimulating the dissociation of GDP from Cdc42Hs. Submitted to *J. Biol. Chem.*
5. Friedberg, I., Cerione, R.A., and Gamett, D.C. Role of secondary dimerization of class 1 receptor tyrosine kinases. In preparation.

## PERSONNEL INVOLVED IN THE STUDIES

| | | | |
|---|---|---|---|
| 1. | Richard A. Cerione | Ph.D. | P.I. |
| 2. | Daniel C. Gamett | Ph.D. | Postdoctoral Associate |
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## MEETING PRESENTATIONS 6/15/95-6/15/96-

1. Friedberg, I., Cerione, R.A., and Gamett, D.C. The importance of secondary dimerization of class 1 receptor tyrosine kianses in growth factor signaling. "Oncogene meeting" in Frederick, Maryland.
2. Cerione, R.A. Identification of regulatory proteins for Cdc42, a Ras-like protein involved in cell growth and cytokinesis. "Meeting for the American Society for Biochemistry and Molecular Biology" in New Orleans, Louisiana.

**Identification of c-Cbl as a Signaling Partner for the EGF receptor and c-Crk in Mammary Cancer Cells.**

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## Abstract

In this work, we set out to identify proteins in human breast cancer cells (MDA-468 and SKBR5 cells) that were phosphorylated in an EGF-dependent and/or heregulin-dependent manner. We found that an ~130 kDa protein (often appearing as a doublet), which associated with the SH2 and SH3 domains of c-Src and with the amino terminal SH3 domain of Grb-2, was a predominant tyrosine phospho-substrate following the treatment of breast cancer cells with EGF. The phosphorylation of this protein (p130) is specifically stimulated by EGF and not by heregulin, suggesting that it is not a phospho-substrate for the activated Neu/ErbB2 tyrosine kinase, but apparently is a specific phospho-substrate for the EGF receptor. The EGF-dependent phosphorylation of p130 was rapid and could be observed within 1 minute of growth factor treatment. The ability of p130 to bind to Grb-2 suggests that it may be recruited to the EGF receptor via this adapter molecule. We ruled out a number of candidate proteins in the 120-130 kDa range as being identical to p130, including the Ras-GTPase-activating protein (Ras-GAP), the focal adhesion kinase (FAK), and the Crk-associated Src-substrate, Cas. However, Western blot analyses and co-immunoprecipitation experiments were consistent with p130 being identical to c-Cbl, which is the proto-oncogenic product of the transforming gene of the Casitas B-lineage Lymphoma NS-1 retrovirus. We find that c-Cbl will bind to c-Crk in breast cancer cells in an EGF-dependent manner and that this association is rapid and correlates with the EGF-dependent phosphorylation of Cbl. The binding of Cbl to Crk occurs through the SH2 domain of Crk and appears to be accompanied by the dissociation of Crk from the EGF receptor. Overall, these findings lead us to propose that Cbl is initially recruited to the EGF receptor via the Grb-2 protein and that following its phosphorylation, Cbl associates with Crk and then the Cbl--Crk complex dissociates from the EGF receptor, possibly to initiate some type of signaling event.

## Introduction

The epidermal growth factor receptor (EGFR) is activated by binding multiple ligands, the most important of which are epidermal growth factor (EGF) and transforming growth factor-$\alpha$ (TGF$\alpha$) (1). EGF or TGF$\alpha$ binding greatly enhances the catalytic activity of the EGFR which undergoes autophosphorylation and phosphorylates numerous intracellular substrates (2). As will be described below, we have recently identified an ~130 kDa EGF-stimulated phospho-protein in human breast cancer cells as the Cbl proto-oncogene product. Cbl is a newly described EGFR substrate (3,4) that contains a nuclear localization sequence in the amino terminal-third of its amino acid sequence. The carboxyl two-thirds of c-Cbl contains a RING finger (zinc-binding motif), a leucine zipper, a domain that contains a high proportion (19%) of serine and threonine residues, 17 proline-rich motifs, and several tyrosine residues (5). It originally was suspected that Cbl may have a hematopoietic-specific function and has been shown to be tyrosine phosphorylated in Jurkat T cells after T cell receptor cross-linking, as well as transiently tyrosine phosphorylated in macrophages after CSF-1 stimulation (6,7). However, recent findings suggest that c-Cbl may play a more general role in EGFR signaling in mammalian cell (3,4,8), although its specific nature is not known.

A good deal of effort has gone into studying cellular transformation by the viral homolog of Cbl. The *v-cbl* oncogene is the transforming gene of the Cas NS-1 murine lymphoma virus (5) and encodes a viral Gag sequence fused to the 355 amino-terminal residues of c-Cbl. The carboxy-truncated v-Cbl lacks the proline-rich domain, the serine/threonine-rich domain, the RING finger and the leucine zipper of c-Cbl. The viral oncogene product is localized to both the cytoplasm and the nucleus (9). The proteins encoded by *v-cbl* or a DNA construct containing the 360 amino-terminal amino acids of c-Cbl (c/v-Cbl) bind DNA, while the c-Cbl protein does not (9). Overexpressed c-Cbl does not transform NIH-3T3 cells or induce tumor growth. These results suggest that the carboxy-terminal two-thirds of c-Cbl prevent nuclear localization of the amino-terminal third and that nuclear localization (as well as DNA binding) are essential for

transformation by v-Cbl. However, the c-Cbl protein becomes transforming after deletion of 17-amino acids (i.e. residues 366-382) carboxy-terminal to the v-Cbl coding region (10). This region appears to be extremely important for maintenance of normal c-Cbl function since deletion of a single amino acid in this region is sufficient to activate c-Cbl to induce transformation. The protein encoded by the 17-amino acid deletion is retained in the cytoplasm, but it is highly tyrosine phosphorylated. Thus, it is suspected that hyper-phosphorylation of Cbl may cause it to become over-active in some type of signaling pathway, thereby triggering transformation. It has been proposed that in some cases, this aberrant tyrosine phosphorylation is catalyzed by the Abl tyrosine kinase (10). Interestingly, v-Cbl is not highly tyrosine phosphorylated. Therefore, it is likely that v-Cbl and the 17-amino acid deletion mutant of c-Cbl transform cells via different mechanisms.

While investigating the EGFR signaling pathway in breast cancer cell lines, we found that upon the EGF-stimulated (tyrosine) phosphorylation of c-Cbl, it associated with c-Crk, a proto-oncogene product that codes for a 40 kDa cytoplasmic adaptor protein which contains an amino terminal SH2 domain followed by two SH3 domains (11-13). Unexpectedly, we also found that c-Crk appeared to be constitutively associated with the EGFR and that a fraction of c-Crk dissociated from the EGFR after EGF stimulation. This dissociation appeared to be correlated with the EGF-promoted binding of c-Cbl to c-Crk. The apparent migration of c-Crk from the EGFR to c-Cbl may represent a novel mechanism of signal transduction whereby a small adaptor protein like c-Crk associates with a larger putative adaptor protein (c-Cbl), thereby increasing the spectrum of potential protein interactions that participate in EGF-coupled signaling.

## Materials and Methods

### Cell culture and antibodies

MDA-MB 468 human mammary carcinoma cells were kindly provided by Mark Sliwkowski (Genentech). The SKBR3 human mammary carcinoma cells were purchased from ATCC. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum. Subconfluent culture plates were switched to serum-free DMEM 40 hours prior to growth factor stimulation. Cells were stimulated with EGF (Calbiochem) or with the EGF-like domain of heregulin β1 (residues 177 to 244; a gift from Mark Sliwkowski, Genentech) diluted in serum-free DMEM at 37°C at the concentrations and the durations indicated. Monoclonal anti-phosphotyrosine antibody, PY20, and anti-Crk monoclonal antibody were obtained from Transduction Labs (Lexington, KY). Polyclonal anti-Cbl and anti-EGFR antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GST-Crk-SH3 and GST-Crk-SH2 fusion proteins were kindly provided by Beatrice Knudsen of Rockefeller University. GST-Src-SH3, GST-Src-SH2 and GST-Grb2-SH3 fusion proteins were a gift from Dr. Steven Taylor of Cornell University.

### Immunoprecipitation and immunoblotting

Cells were washed twice with ice cold phosphate-buffered saline containing 1 mM sodium vanadate and lysed with NP40 lysis buffer (1% NP40, 20 mM Tris-hydrochloride [pH 8.0], 150 mM NaCl, 25 mM NaF, 1 mM EDTA, 1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 15 minutes at 4°C. Lysates were centrifuged at 13,000 X g for 20 min at 4°C and the proteins in the supernatant were quantified by the Lowry method (Bio-Rad DC Protein Assay). The lysates were pre-cleared with 25 μl of 50% Protein A-Sepharose beads (Sigma) for one hour prior to immunoprecipitation. One milligram of protein from each culture dish was immunoprecipitated for one hour with antibody followed by adsorption to 25 μl of 50% Protein A-Sepharose beads at 4°C for one hour. Lysates used for GST-fusion protein precipitation were pre-

cleared by incubation with 25 μl of 50% glutathione-agarose beads (Sigma). One milligram of lysate protein was immunoprecipitated with 10 μg of fusion protein bound to 25 μl of 50% glutathione-agarose beads for one hour. In all cases, the precipitates were washed three times with NP40 lysis buffer and then boiled for 5 minutes in sample buffer containing 50 mM Tris (pH 6.7), 2% SDS, 2% β-mercaptoethanol, and bromophenol blue. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes for Western blotting.

The PVDF membranes were blocked with 2% nonfat dry milk powder in Tris-buffered saline (pH 7.4) plus 0.1% Tween 20 (TBS-Tween) for one hour at room temperature. The blots were then immunolabeled by incubation with monoclonal or polyclonal antibody diluted in TBS-Tween at room temperature followed by washing with several changes of TBS-Tween for 30 minutes. The blots were then incubated with sheep-anti-rabbit or rabbit-anti-mouse-horseradish peroxidase-conjugated antibody (Amersham) for one hour at room temperature and washed. Immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's instructions. Blots were stripped for reprobing with a second antibody by incubating in a solution containing 2% SDS and 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8).

## Results

### *GST-Src-SH3 and -SH2 bind to an ~130 kDa tyrosine phosphorylated protein in mammary carcinoma cells stimulated with EGF-*

During an initial screening for novel EGFR substrates in MDA-MB-468 human mammary carcinoma cell lysates, we found a 130 kDa protein (p130) that became highly tyrosine phosphorylated and bound to GST-Src-SH3 and GST-Src-SH2 fusion proteins (Figure 1A). Note that an 180 kDa protein also was associated with GST-Src-SH2; we have determined that this is the (auto)phosphorylated EGF receptor. When performing similar experiments with the amino-terminal SH3 domain of Grb-2, we found that p130 also appeared to associate with this adaptor molecule (Figure 1B). In order to determine the identity of p130, we probed blots with antibodies against several 120-130 kDa proteins. Two 130 kDa proteins, c-Cbl and c-Cas (14,15) could be precipitated with both GST-Src-SH2 and GST-Src-SH3 fusion proteins (data not shown). However, only the c-Cbl protein bound to GST-Grb2-SH3 (compare Figures 2A and 2B) and was tyrosine phosphorylated after EGF stimulation (Figure 2C). Moreover, unlike Cas, c-Cbl was not detected to co-immunoprecipitate with anti-Src antibody (data not shown). Thus, we have concluded that p130 is the Cbl proto-oncogene product.

EGF stimulation induced tyrosine phosphorylation of c-Cbl in another breast cancer cell line, SKBR3. These cells overexpress EGFR and two EGFR family members, the Neu/ErbB2 and ErbB3 proteins (16,17). Figure 3 shows that when SKBR3 cells were treated with heregulin, a ligand for ErbB3 and ErbB4 and an activator of Neu tyrosine kinase activity, presumably through Neu--ErbB3 and Neu--ErbB4 heterodimer formation (18,19), a major phospho-band at ~185 kDa was observed (most likely Neu) but no phosphorylation of c-Cbl was detected.

Since the phosphorylation of c-Cbl was specifically stimulated by EGF, we examined whether the (EGF-stimulated) phosphorylation of c-Cbl could be detected in MDA-MB-453 human mammary carcinoma cells, which do not overexpress EGFR. Although c-Cbl is expressed at

roughly equivalent levels in MDA-MB-468 and SKBR3 cells, there was no evidence of EGF-induced tyrosine phosphorylation of Cbl in MDA-MB-453 cells (data not shown).

***Time- and dose-dependence of c-Cbl phosphorylation-***

In order to determine the kinetics of the EGF-stimulated phosphorylation of c-Cbl, we stimulated MDA-MB-468 cells with EGF for various time periods before lysis and anti-Cbl immunoprecipitation. Anti-phosphotyrosine immunoblotting showed that tyrosine phosphorylation of c-Cbl occurred within 2 minutes of EGF stimulation and persisted for at least 60 minutes (Figure 4A). The blot was stripped and re-probed with anti-Cbl antibody to demonstrate equal loading of c-Cbl in each lane of the gel (Figure 4B). In Figure 4A, the band migrating at 180 kDa is the EGFR. Thus as has been reported for NIH-3T3 cells over-expressing EGFR (3), it is possible that Cbl can directly associate with the EGFR in breast cancer cells. However, when we first immunoprecipitate the EGFR and then Western blot with anti-phosphotyrosine antibodies (Figure 4C), we typically detect little if any associated Cbl. This may mean that the association of Cbl with the EGFR is relatively weak or that the anti-EGFR used for immunoprecipitation in some manner perturbed the interaction between the EGFR and Cbl. The results in Figure 4C also show the EGF-stimulated phosphorylation of the EGFR occurred on the same time scale as the phosphorylation of c-Cbl and again persisted through 1 hour.

The EGF-induced tyrosine phosphorylation of c-Cbl was also dose-dependent. Figure 4D shows that the EGF-stimulated phosphorylation was maximal when the cells were incubated with 100 ng/ml of EGF.

***c-Cbl associates with the c-Crk adaptor protein in an EGF-dependent manner-***

During our initial investigation of EGFR signaling, we considered that p130 might be the c-Src-associated protein, Cas (14,15). Since c-Cas has numerous Crk-SH2 consensus binding sites and is tyrosine phosphorylated in v-Crk transformed cells, we first tested whether p130 bound to the c-Crk adaptor protein. As shown in Figure 5A, we found that a 130 kDa phosphoprotein co-

immunoprecipitated with c-Crk in an EGF-dependent manner. Although Cas was present in the MDA-MB-468 cells, it weakly co-immunoprecipitated with c-Crk in an EGF-independent manner and it did not become tyrosine phosphorylated after EGF stimulation (Figure 5B). When MDA-MB-468 lysates were immunoprecipitated with anti-phosphotyrosine and anti-Crk antibodies and probed with anti-Cbl antibody, we found that c-Cbl was tyrosine phosphorylated and associated with c-Crk in an EGF-dependent manner (Figure 5C). To determine which domain of c-Crk was necessary for the c-Crk/c-Cbl interaction, we precipitated MDA-MB-468 lysates with GST-Crk-SH2 and GST-Crk-SH3 fusion proteins. Lysates were also immunoprecipitated with anti-Crk antibody and then probed with anti-Cbl antibody. The results presented in Figure 5D show that c-Cbl associated with the Crk-SH2 domain in an EGF-dependent manner, whereas no interaction was detected between Cbl and the GST-Crk-SH3 fusion protein.

***c-Crk dissociates from the EGFR and associates with c-Cbl after EGF stimulation-***

Since we had shown that c-Crk associated with c-Cbl in an EGF-dependent manner, we investigated the association between the EGFR and c-Crk during the time course for EGF-stimulated phosphorylation of Cbl. To do this, we first immunoprecipitated c-Crk and then Western blotted with an anti-EGFR antibody. The results shown in Figure 6A suggest that c-Crk is constitutively associated with the EGFR in MDA-MB-468 cells. After EGF stimulation, the amount of c-Crk that was co-immunoprecipitated with the EGFR was reduced (i.e. after 2 minutes). Thus, apparently some fraction of the total pool of c-Crk that was initially bound to the EGFR dissociated from the receptor upon EGF stimulation. We have tried to determine whether Cbl also dissociates from the EGFR under conditions where the dissociation of c-Crk from the receptor occurs; but thus far, we have not been able to reach a definitive conclusion from experiments where anti-EGFR precipitates are blotted with anti-Cbl antibody. However, Figure 6B shows that under identical experimental conditions as those described in Figure 6A, the interaction of c-Cbl with c-Crk was stimulated by a short (2 minute) treatment with EGF, as

monitored by Western blotting anti-Crk immunoprecipitates with anti-Cbl antibody. This Cbl--Crk complex appeared to be maintained through 1 hour.

It is interesting that there appear to be two forms of c-Crk in MDA-MB-468 cells. When whole cell lysates were immunoprecipitated and blotted for c-Crk, a protein doublet migrating at approximately MW 40,000 was detected (Figure 7A). Serum-starved cells contained a greater proportion of the more rapidly migrating form. After EGF stimulation, the more slowly migrating form predominated. The same band shift was apparent when anti-Crk immunoprecipitates were probed with anti-phosphotyrosine antibody (Fig 7B). This suggests that the slower migrating band represents a more highly tyrosine phosphorylated form of c-Crk that becomes prevalent after EGF stimulation and remains phosphorylated for at least 60 minutes. This EGF-stimulated phosphorylation may influence (promote) Crk interactions with Cbl and/or the dissociation of Crk from the EGF receptor.

## Discussion

In these studies, we have shown that the Cbl proto-oncogene product is a predominant (tyrosine) phospho-substrate for the EGFR in human breast cancer cells. This phosphorylation is specifically stimulated by EGF and is not observed following heregulin treatment of cells. Thus, it is likely that Cbl interacts with the EGFR and is incapable of coupling to heterodimer combinations of Neu/ErbB2 and ErbB3 or Neu/ErbB2 and ErbB4. The specific mechanism underlying the coupling of Cbl to activated EGF receptors in breast cancer cells is not known, although it is likely to be mediated, at least in part, by the Grb-2 adaptor molecule. The amino-terminal SH3 domain of Grb-2 associates with Cbl in an EGF-independent manner, whereas the SH2 domain of Grb-2 binds to a phospho-tyrosine residue within the carboxyl terminus of the EGFR (20). Thus, it seems likely that EGF-stimulated activation of the EGFR can recruit a Grb-2--Cbl complex, resulting in the phosphorylation of Cbl.

An interesting outcome of these studies is the observation that the EGF-stimulated phosphorylation of Cbl is accompanied by an EGF-dependent association of Cbl with the Crk proto-oncogene product. Deletion of the carboxyl-terminal SH3 domain of Crk results in cellular transformation (21) and it has been proposed that this may be the outcome of excess tyrosine phosphorylation mediated by the interactions of Crk with the Abl tyrosine kinase (22). However, the normal role that c-Crk plays in growth factor-coupled signaling is unknown, and thus the finding that c-Cbl binds to c-Crk in an EGF-dependent manner suggests some interesting implications regarding the participation of Crk in the action of the EGFR (see below). Our findings suggest that the Cbl--Crk interaction may be mediated by a prior association of c-Crk with the EGF receptor in human breast cancer cells. To our knowledge, there have been no previous reports of *in vivo* binding of c-Crk with the EGFR. It is possible that the EGFR--Crk association is a specific characteristic of the MD-MB-468 breast cancer cells, since neither constitutive binding of c-Crk to the EGFR, nor EGF-induced tyrosine phosphorylation of c-Crk was detected in A431 cells (23). Both A431 cells and MDA-MB-468 cells express high levels of EGFR (approximately

2 X $10^6$ receptors per cell) (23). Thus, if simply high levels of EGFR were necessary to provide detectable EGFR--Crk complexes, there should be little difference between the cell lines. It is, however, possible that the levels of expression of Crk are anomolously high in the breast cancer cells and thus give rise to its association with the EGFR. This might also explain the finding that the EGFR--Crk association is detectable even under conditions of serum starvation (that is the combination of high levels of EGFR and Crk could allow EGFR--Crk interactions to occur even in the absence of added growth factor). The interaction of Crk with the EGFR appears to be accompanied by the phosphorylation of Crk on a tyrosine(s) residue.

The time course for the EGF-stimulated phosphorylation of Cbl appears to correlate with the interaction between Cbl and c-Crk. This interaction apparently occurs through the Crk-SH2 domain and may result in the dissociation of Crk from the EGF receptor. Taken together, these findings are then consistent with a scheme where the phosphorylation of Cbl results in its uncoupling from the EGF receptor and its association with Crk, which in turn leads to the formation of distinct (Crk--Cbl) complex. In some ways, this is reminiscent of what we observed with ErbB3 and the 85 kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase (24). In this case, heregulin first stimulated the formation of a complex between the Neu/ErbB2 tyrosine kinase and the ErbB3 protein, which is kinase-defective (25). The resulting trans-phosphorylation of ErbB3 then led to its dissociation from Neu/ErbB2 and promoted the formation of a new complex between ErbB3 and p85.

An obviously important question concerns whether the formation of an EGF-stimulated Cbl--Crk complex has important signaling implications. It recently was shown that Cbl interacts with p85 in an EGF-dependent manner in A431 cells and it was proposed that Cbl may serve as an interface or adapter molecule for EGF effects on PI 3-kinase in these cells (26). In a similar vein, Cbl may act to interface Crk and Crk-binding proteins (e.g. Abl) with the EGF receptor in mammary epithelial cells. However, it also is possible that Cbl plays some type negative-regulatory role in the actions of the EGF receptor (27). This suggestion is based on the findings that the homolog for c-Cbl in *C. elegans* is the Sli-1 protein, which has been reported to antagonize

EGF-stimulated signaling (27). Thus, one possibility is that the EGF-stimulated phosphorylation of Cbl enables it to interact with other signaling molecules that normally couple to the EGF receptor and mediate some aspect of EGF-stimulated signaling. For example, the binding of phosphorylated Cbl to c-Crk may prevent other signaling molecules from associating with Crk (i.e. through a competitive mechanism) and thereby have a negative effect on Crk signaling. Future studies will be directed toward examining these possibilities and in particular determining the identity of cellular proteins that associate with, or are regulated as an outcome of, EGF-stimulated Cbl--Crk interactions.

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## Legends

Fig 1. **An 130 kDa EGF-dependent tyrosine phosphorylated protein is detected in human breast cancer cells.** MDA-MB-468 cells were serum starved and unstimulated (-) or stimulated (+) with 100 ng/ml of EGF for 10 min. Lysates were incubated with equimolar amounts of GST-fusion proteins immobilized on glutathione agarose beads, washed, and subjected to SDS-PAGE (7.5%) and analyzed by Western blotting with anti-phosphotyrosine antibody. A, Precipitation with GST-Src-SH3, GST-Src-SH2, or GST-agarose. B, Precipitation with GST-Src-SH3, GST-Grb2-SH3 or GST-agarose.

Fig 2. **The c-Cbl proto-oncogene product is the 130 kDa protein tyrosine phosphorylated in response to EGF stimulation.** MDA-MB-468 cells were serum starved and unstimulated (-) or stimulated (+) with 100 ng/ml of EGF for 10 min. A, Lysates were incubated with equimolar amounts of GST-Src-SH3, GST-Grb2-SH3, or GST-agarose, washed, subjected to SDS-PAGE (7.5%) and analyzed by Western blotting with anti-Cas antibody (Transduction Laboratories). B, Same as A, but anti-Cbl antibody was used for Western blotting. C, Lysates were incubated with anti-Cas antibody, GST-Src-SH3 fusion protein bound to glutathione agarose beads, or anti-Cbl antibody, washed, subjected to SDS-PAGE (7.5%) and analyzed by Western blotting with anti-phosphotyrosine antibody. GST-glutathione agarose and protein-A Sepharose controls were performed and were negative.

Fig 3. **Heregulin does not stimulate tyrosine phosphorylation of c-Cbl in SKBR3 mammary cancer cells.** SKBR3 cells were serum starved and unstimulated (-), stimulated with 30 nM heregulin for 10 minutes (H), or stimulated with 100 ng/ml EGF for 10 minutes (E). Lysates were immunoprecipitated and Western blotted with anti-phosphotyrosine antibody.

Fig 4. **EGF-stimulated phosphorylation of c-Cbl is dose- and time-dependent.** A, MDA-MB-468 cells were stimulated with 100 ng/ml of EGF for 0, 2,10,15, 30, and 60 minutes. The lysates were immunoprecipitated and Western blotted with anti-phosphotyrosine antibody. B, The blot from A was stripped and re-probed with anti-Cbl antibody. C, Lysates from MDA-MB-468 cells were immunoprecipitated with anti-EGFR antibody and Western blotted with anti-phosphotyrosine antibody. D, MDA-MB-468 cells were stimulated with 0, 25, 50, 100, 150, and 200 ng/ml EGF for 10 minutes. Lysates were immunoprecipitated with anti-Cbl antibody and blotted with anti-phosphotyrosine antibody

Fig 5. **c-Cbl associates with c-Crk in an EGF-dependent manner.** MDA-MB-468 cells were serum starved and unstimulated (-) or stimulated (+) with 100 ng/ml of EGF for 10 min. A, Lysates were immunoprecipitated with anti-Crk antibody and Western blotted with anti-phosphotyrosine antibody. B, Lysates were immunoprecipitated with anti-Crk or anti-phosphotyrosine antibody and Western blotted with anti-Cas antibody. C, Lysates were immunoprecipitated with anti-Crk or anti-phosphotyrosine antibody and Western blotted with anti-Cbl antibody. D, Lysates were incubated with GST-Src-SH3, GST-Crk-SH2 or GST-Crk-SH3 fusion proteins bound to glutathione-agarose beads or immunoprecipitated with anti-Crk antibody and Western blotted with anti-Cbl antibody.

Fig 6. **c-Crk dissociates from EGFR and associates with tyrosine phosphorylated c-Cbl after EGF stimulation**. MDA-MB-468 cells were stimulated with 100 ng/ml EGF for 0, 2,10,15, 30, and 60 minutes. A, Lysates were immunoprecipitated with anti-Crk antibody and Western blotted with anti-EGFR antibody. B, Lysates were immunoprecipitated with anti-Crk antibody and Western blotted with anti-Cbl antibody.

Fig 7. **Two forms of c-Crk are present in MDA-MB-468 lysates.** MDA-MB-468 cells unstimulated (-) or stimulated (+) with 100 ng/ml of EGF for 10 min. The lysates were

immunoprecipitated with anti-Crk antibody. A, The immunoprecipitates were blotted with anti-Crk antibody. B, Anti-phosphotyrosine antibody was used to Western blot the anti-Crk immunoprecipitates.

Fig. 1A

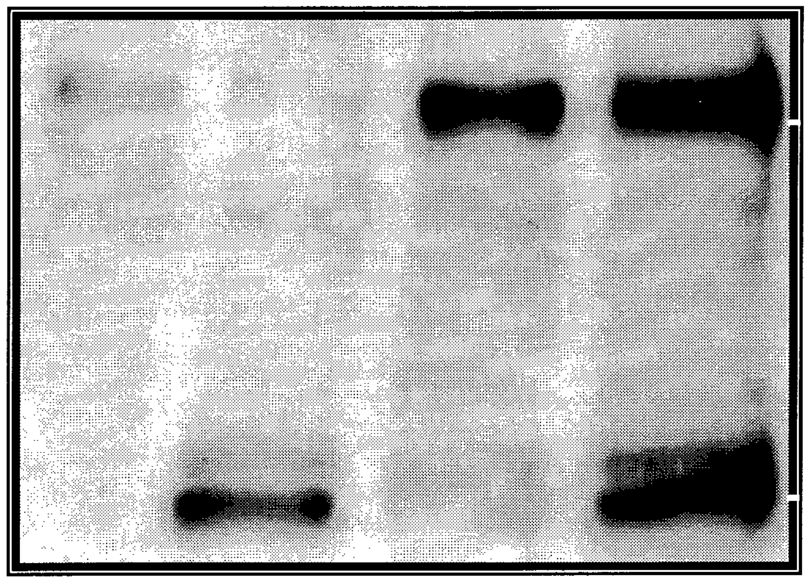


Fig. 1B

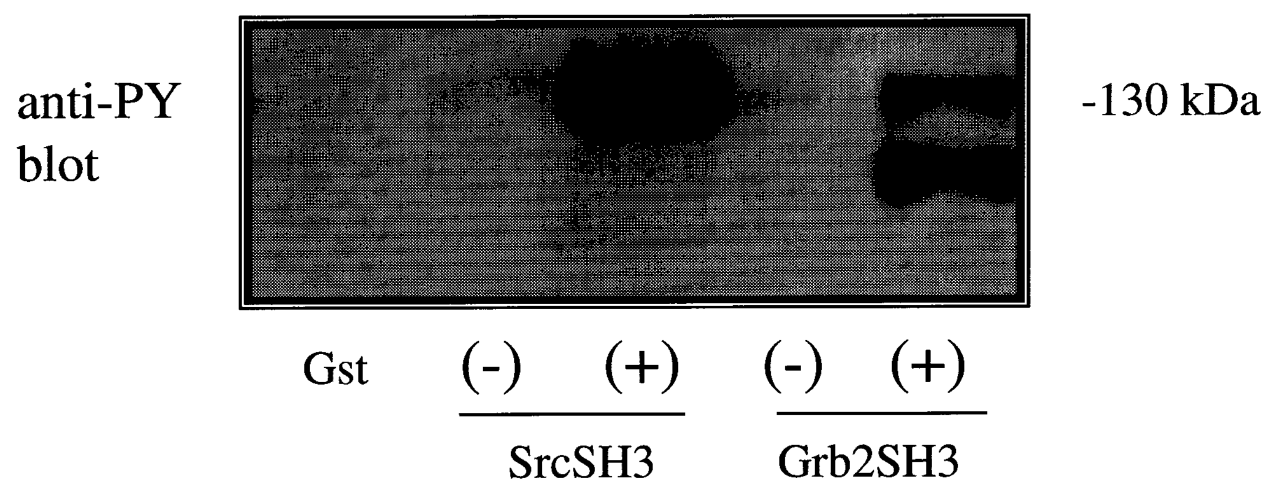


Fig 2A

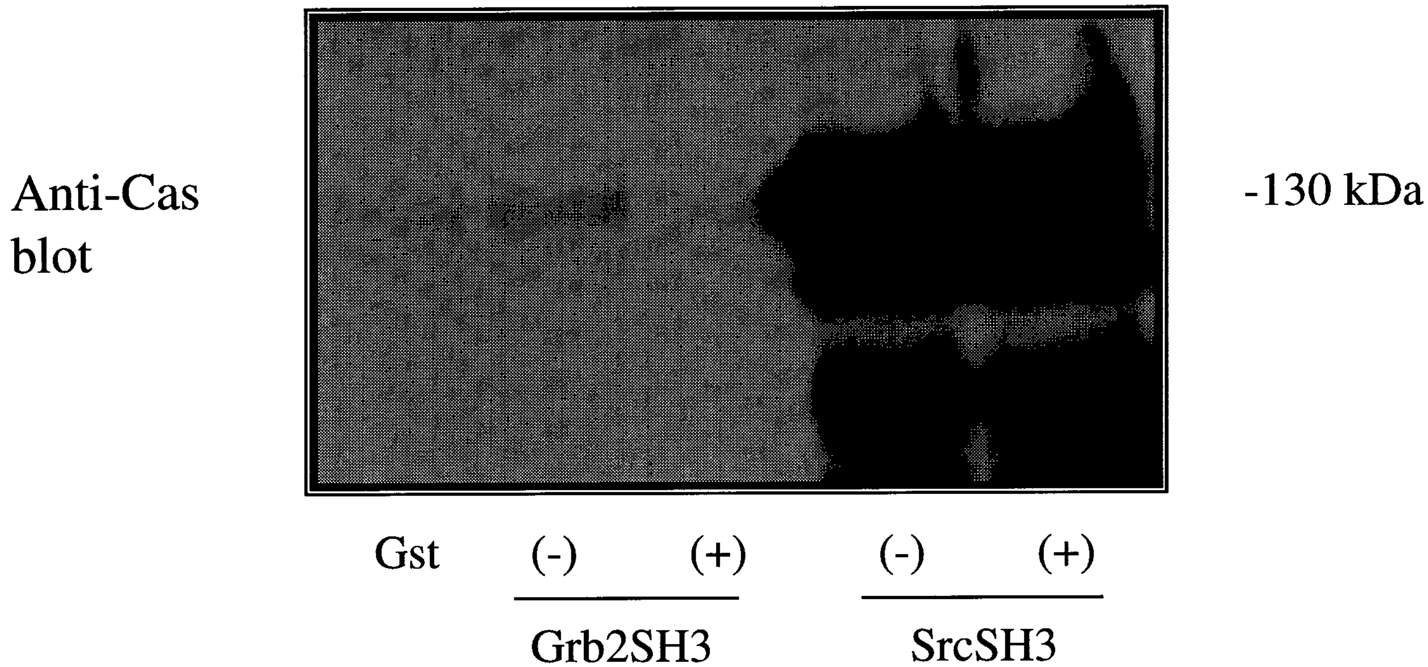


Fig. 2B

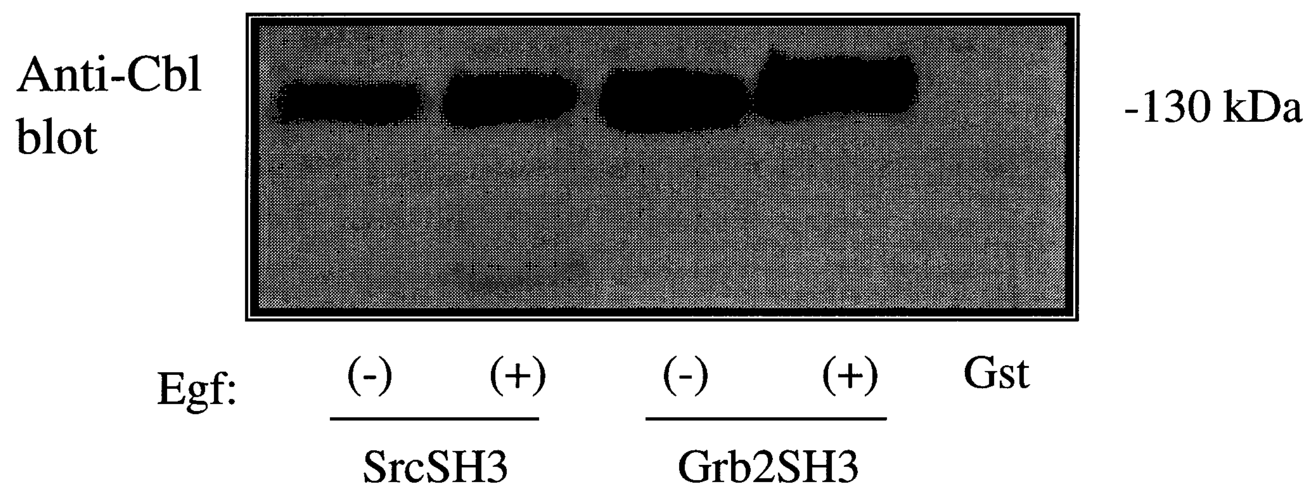


Fig 2C

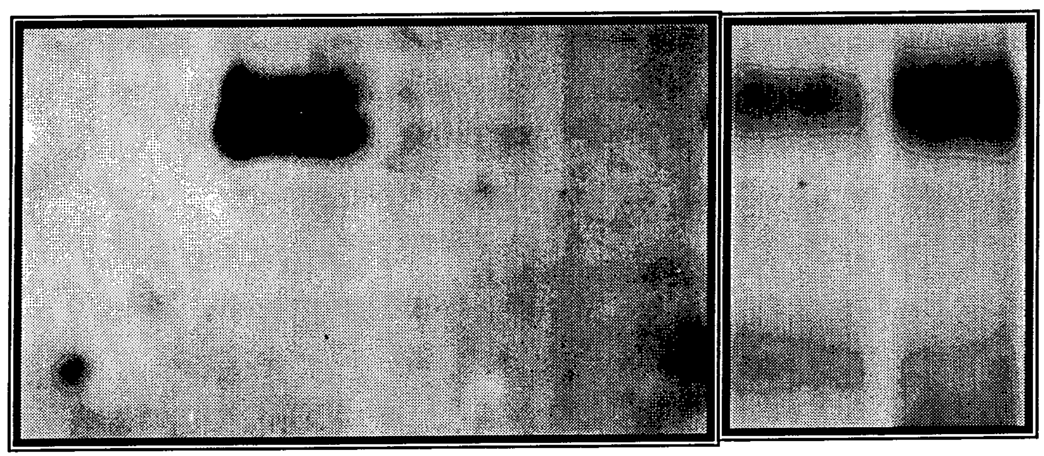


Fig. 3

Fig 4A

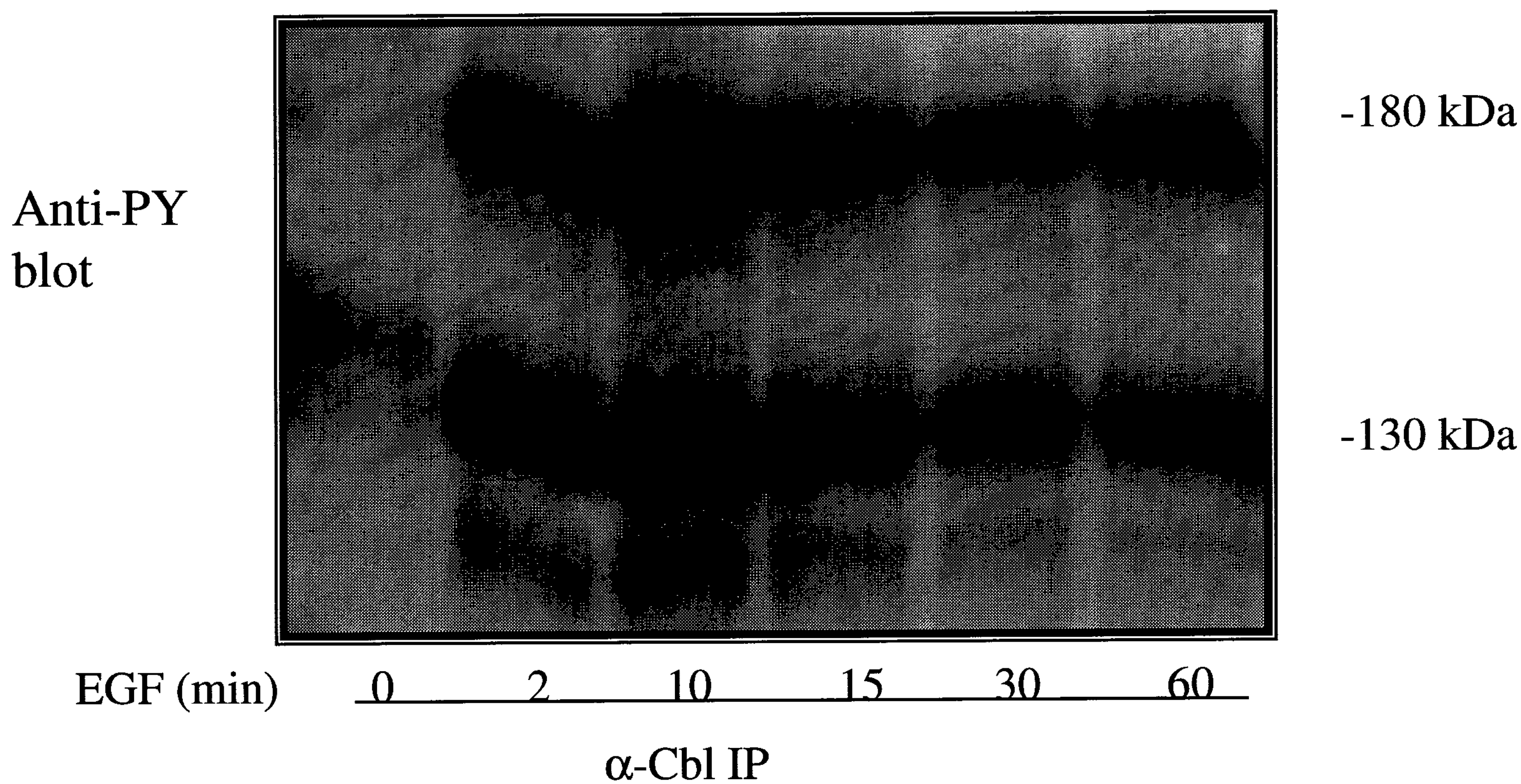


Fig 4B

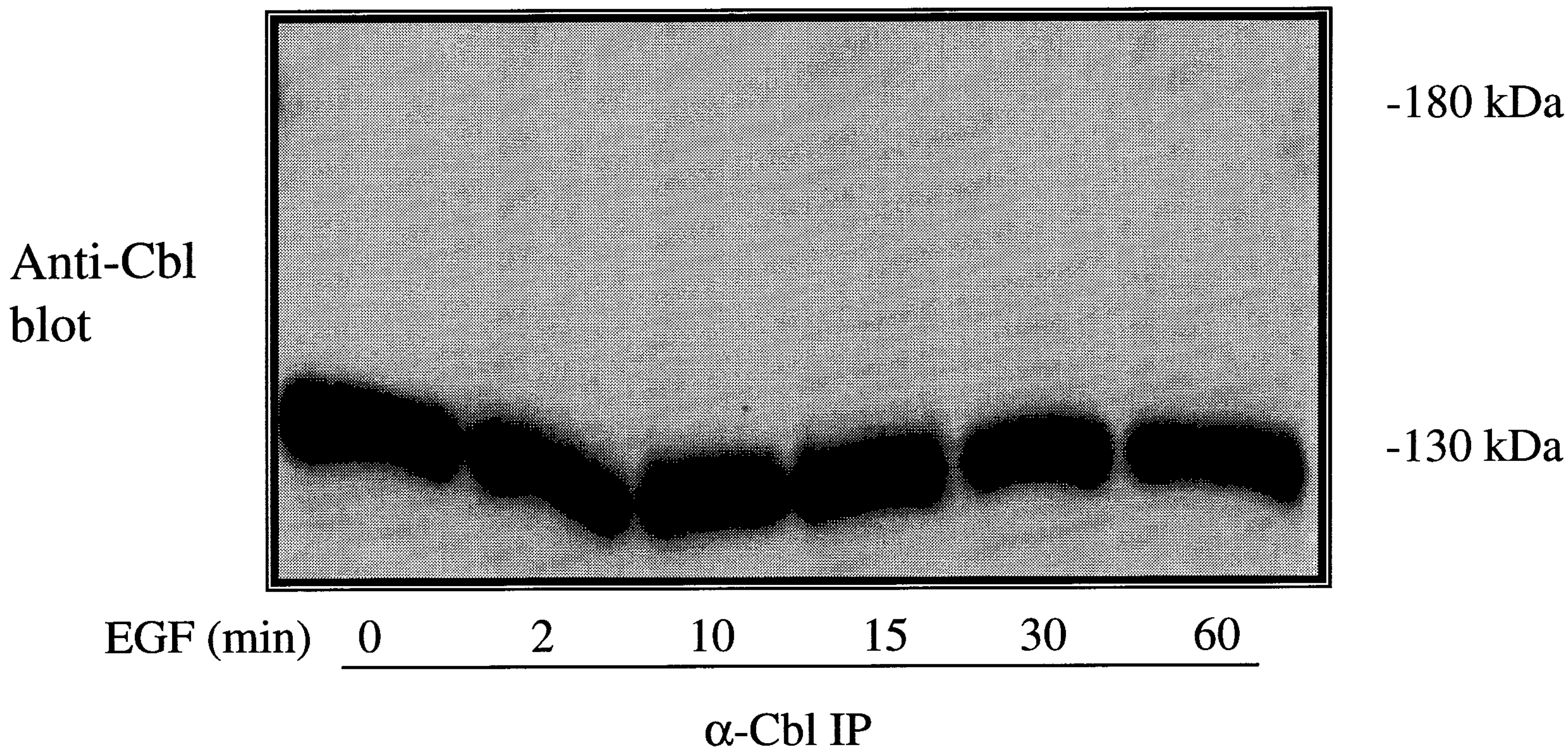


Fig. 4C

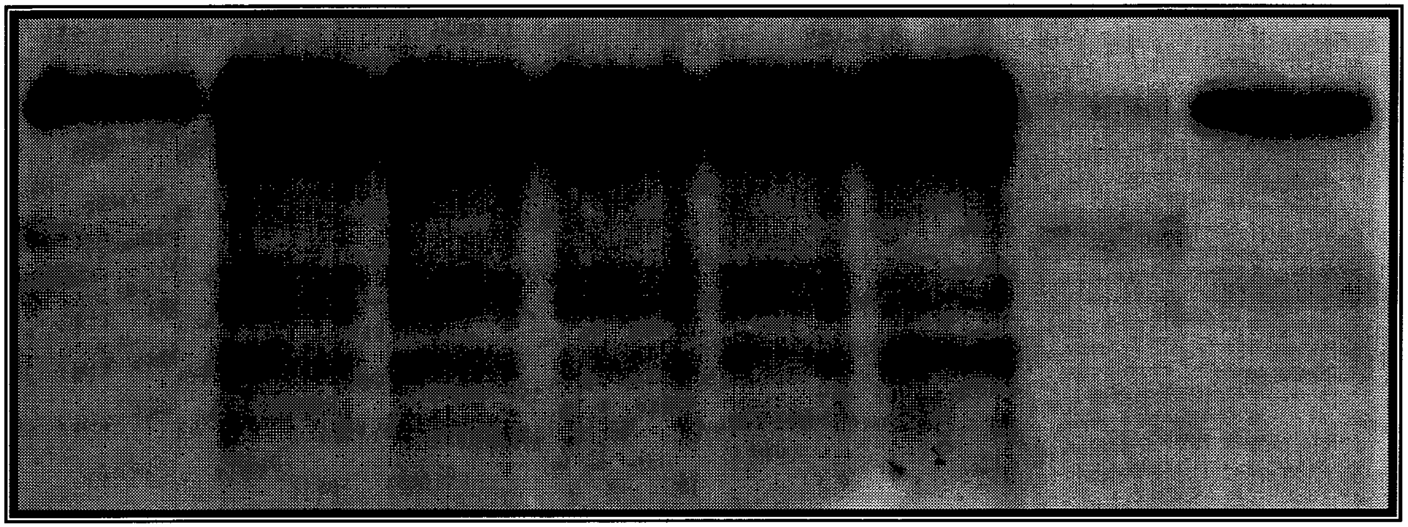

Anti-PY
blot
-180 kdA
-130 kDa
EGF(time): 0 2 10 15 30 60 PGS lys
α-EGFR IP

Fig 4D

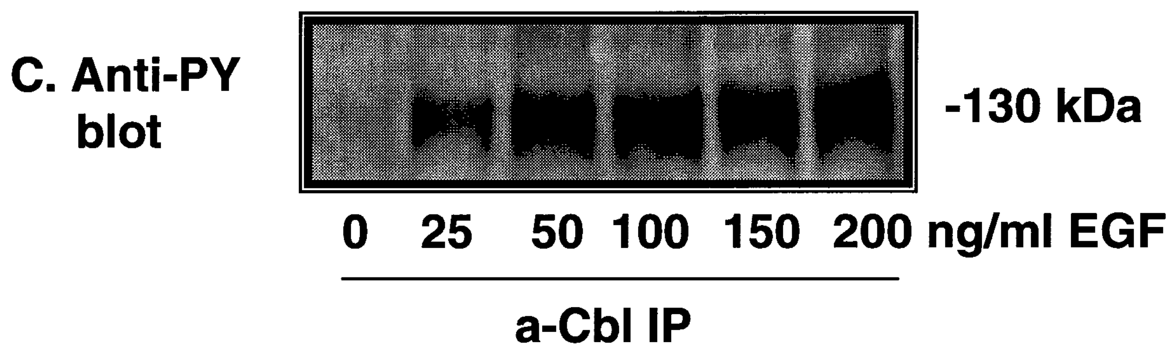


Fig 5A

α-PY blot

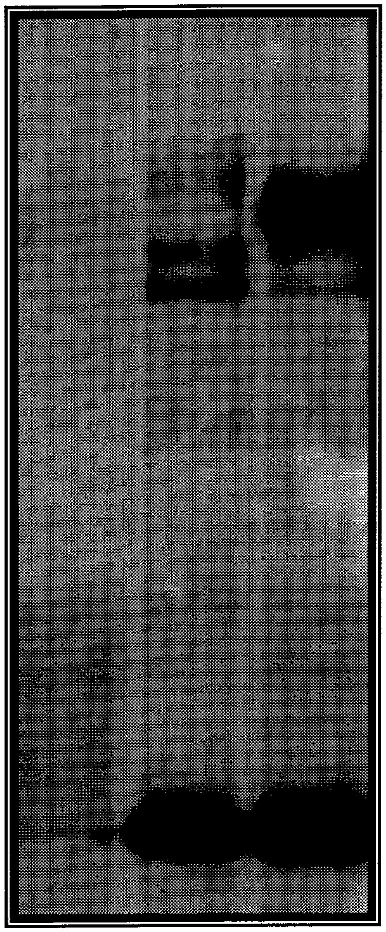



Fig 5B

α-Cas.2 blot

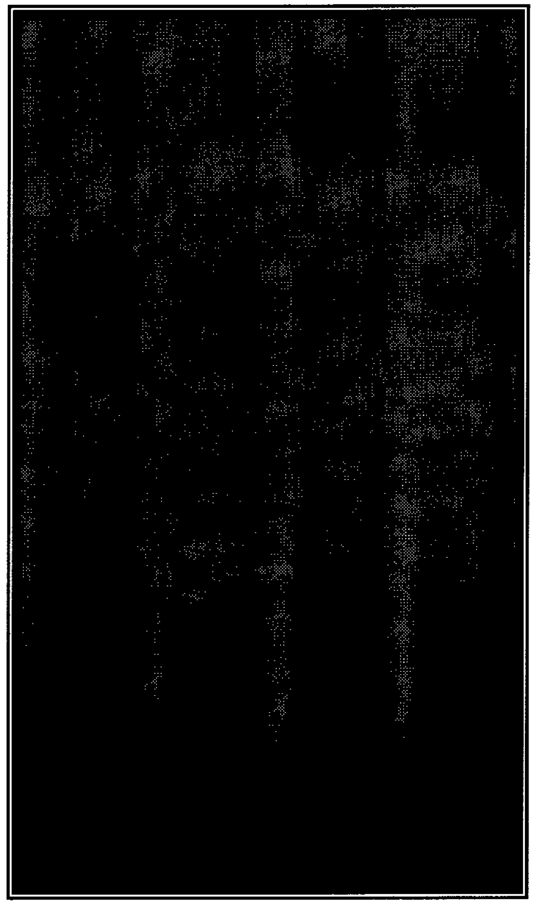



Fig 5C

α-Cbl blot

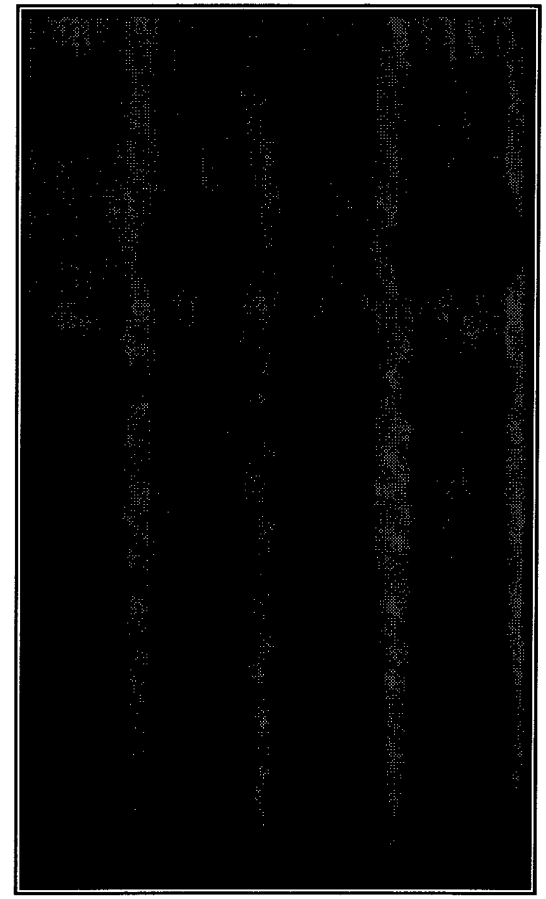


Fig 5D

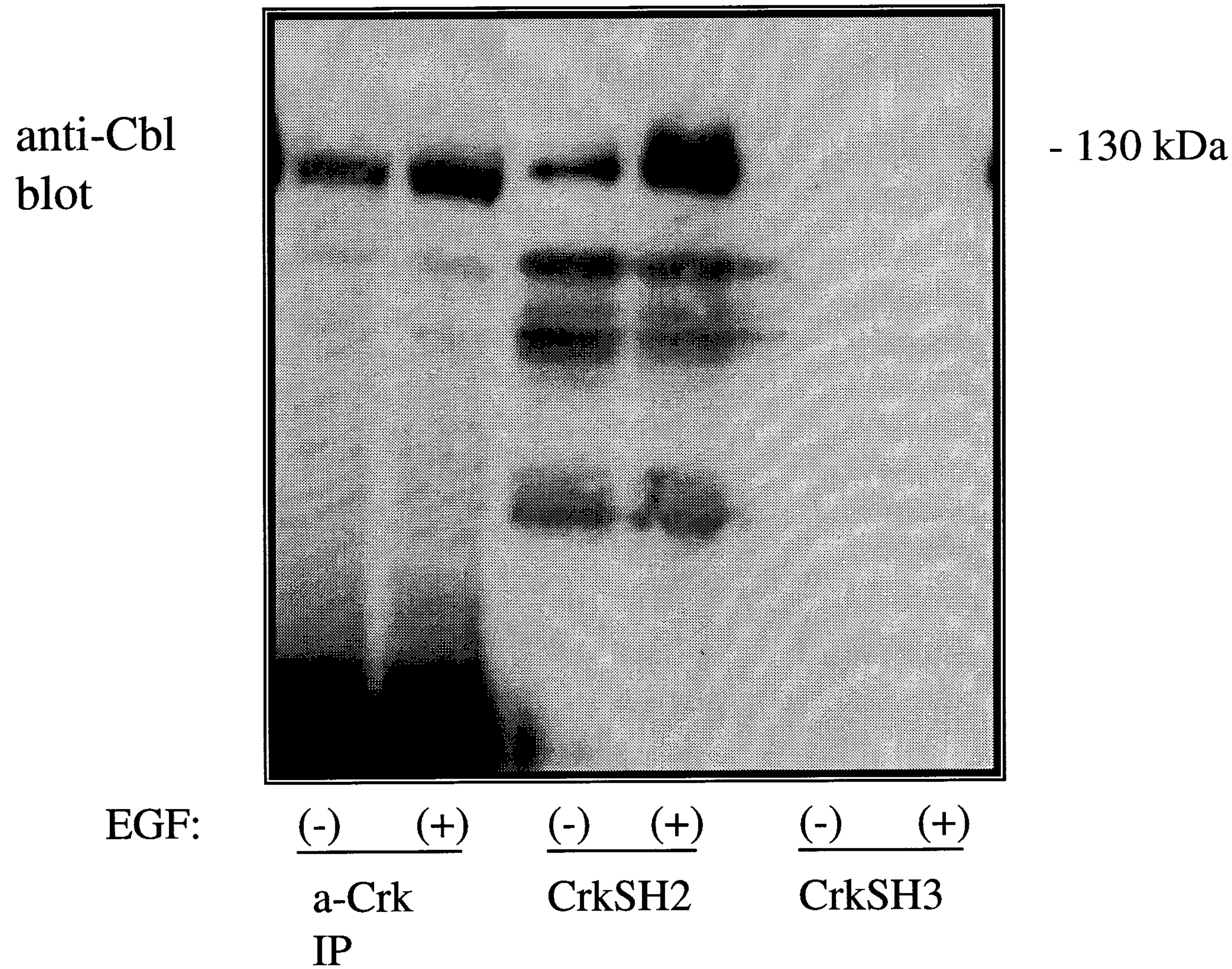
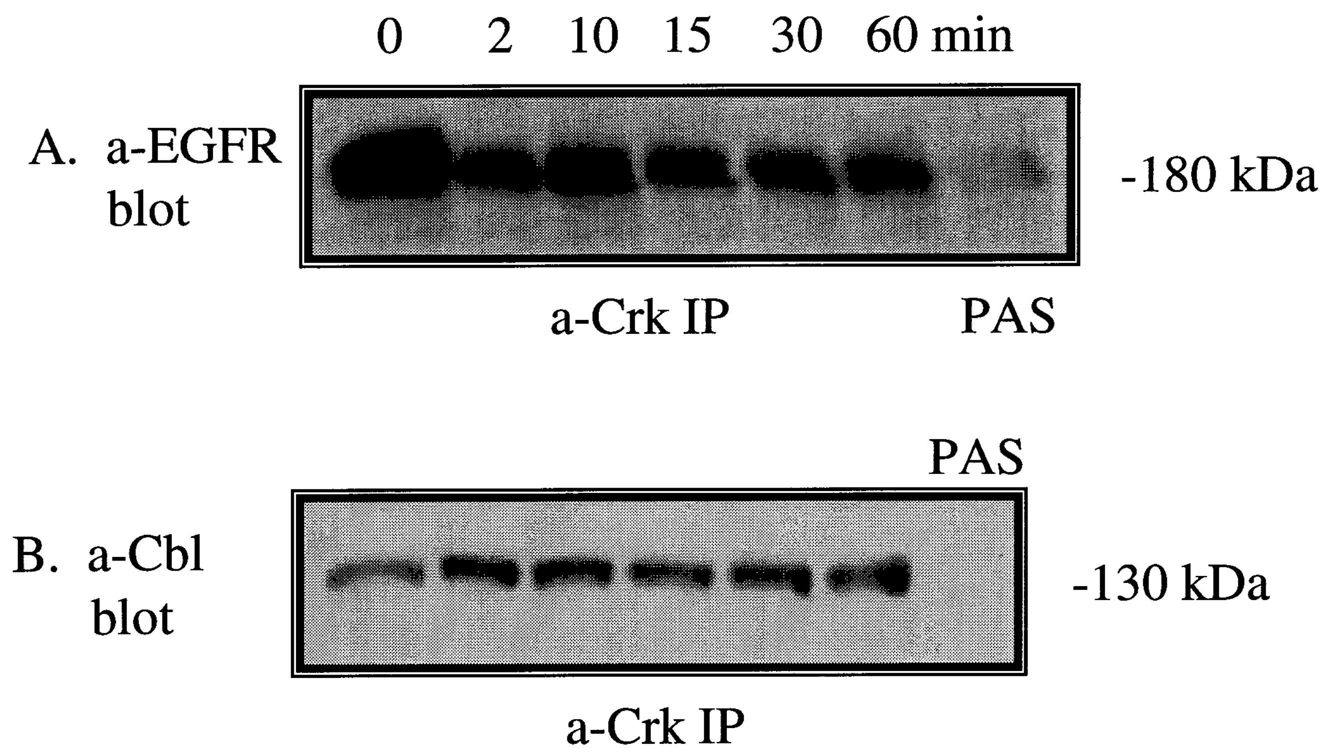
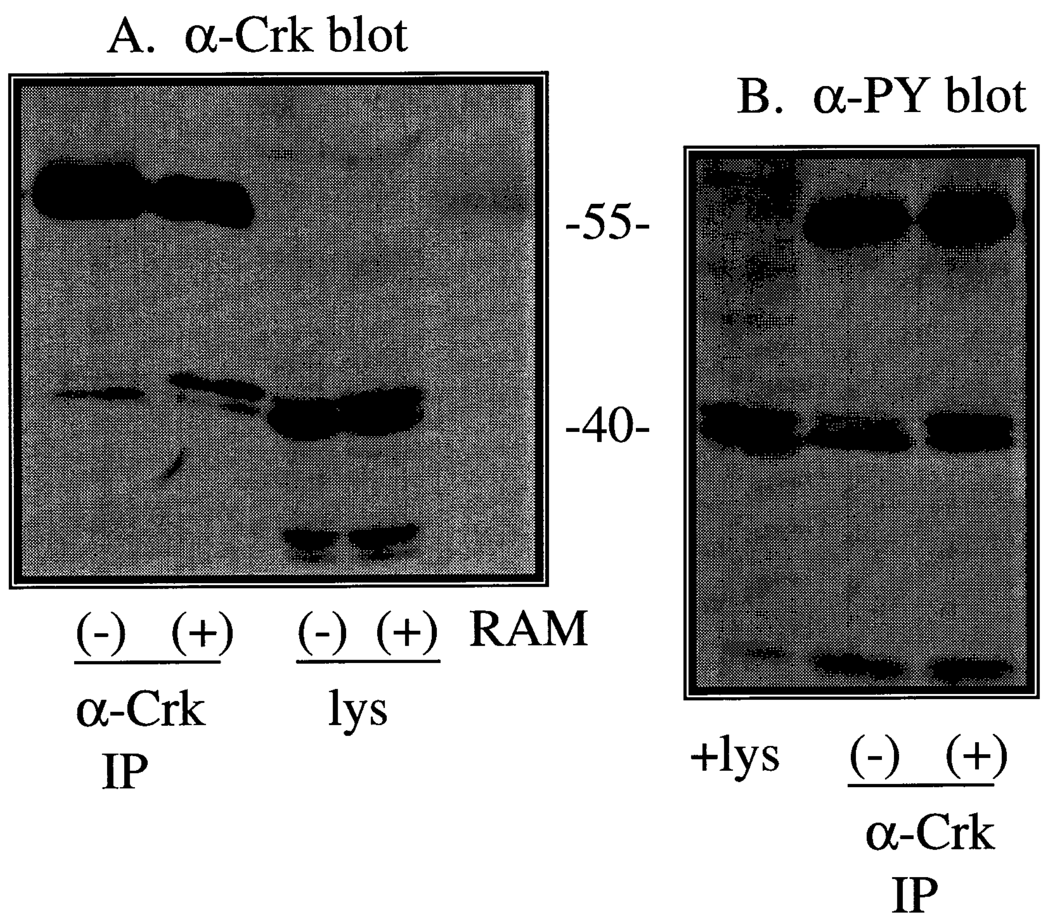


Fig. 6



0 2 10 15 30 60 min
A. a-EGFR blot
-180 kDa
a-Crk IP
PAS
PAS
B. a-Cbl blot
-130 kDa
a-Crk IP

Fig 7



# Identification of a Growth-factor Stimulated GTP-binding Activity in the Nucleus

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GTP-binding proteins are important mediators in growth factor receptor-coupled signal transduction events and in a diverse array of cellular functions. While the role that this superfamily of proteins plays in the cytosol and at the plasma membrane has been an area of intense research, little is known about how these proteins might be involved in nuclear signal transduction. In this report, we identify a specific, nuclear GTP-binding activity which we designate p18 based on its approximate $M_r$ of 18 kDa. p18 responds to growth factor treatment of tissue culture cells with a significant and sustained increase in its ability to bind GTP. Further characterization of this activity revealed a dramatic increase in GTP-binding to p18 in HeLa cells which were arrested in the $G_1$/S phase of the cell cycle, compared with cells arrested in either $G_0$ or M phase. Gel filtration studies indicate that p18 can bind GTP when it is part of a 100-150 kDa complex, but not when it is in a monomeric state. Additionally, the GTP-binding activity of p18 is effected by the presence of the chromatin-associated protein, RCC1, as the loss of RCC1 in tsBN2 cells causes an increase in GTP-binding to p18. These results suggest that p18 may be a mediator of growth factor effects on nuclear functions such as cell-cycle checkpoint control.

# INTRODUCTION

The Ras superfamily of GTP-binding proteins serve as molecular switches crucial to the regulation of numerous cellular events. Members of the superfamily appear to be involved in a number of processes including: cytoskeletal arrangement (Rho subgroup) (1,2), secretion and vesicular trafficking (Rab subgroup) (1), and intracellular transport (Arf subgroup) (3), while Ras itself plays a key, upstream role in mediating growth factor receptor-coupled signal transduction events (4). Such a clear division of labor among subfamilies, however, has recently become more complicated. The Rho subgroup, for example, has now been placed downstream in growth factor-activated pathways mediating cytoskeletal changes (5,6), in the stress-activated PAK/JNK pathways (7,8), and has been coupled with changes in DNA synthesis (7). Given this diversity of function for GTP-binding proteins in the cytosol, it seems reasonable to anticipate a role for GTP-binding proteins in tightly regulated nuclear events such as transcriptional regulation, cell cycle control, and nuclear import/export in response to growth factor stimulation.

To date, only one nuclear GTP-binding protein, Ran/TC4, has been described. Following in suit with other members of the Ras superfamily, Ran has been implicated in a multitude of functions including mRNA processing, nuclear import/export, and cell-cycle checkpoint control (for review, see 9). Whether these activities are coupled or independent of one another remains to be elucidated. Nonetheless, the GTP-binding/GTPase switch activity of Ran appears to be crucial for these nuclear activities to occur in a regulated manner. For example, at START, which represents the commitment of the cell to DNA replication, Ran must switch into its GTP-bound state. Recent data suggest that the guanine nucleotide-exchange factor for Ran, RCC1 (Regulator of Chromatin Condensation) (10) becomes activated to stimulate GDP dissociation from Ran, thereby facilitating its conversion to the GTP-bound (active) state at the onset of S phase. The GTP-bound Ran has been suggested to inhibit the activation of the mitosis/maturation-

promoting factor, such that the GEF activity of RCC1, by activating Ran, serves as a monitor for DNA synthesis initiation and/or progression (11,12). FINISH then would occur as an outcome of the inactivation of RCC1, and the activation of a GTPase-activating protein (GAP) for Ran (13,14), returning it to the GDP-bound form.

Although the activities of Ran and RCC1 have recently become an area of intense research, little is known about the upstream events which may regulate these activities. In particular, not much is understood about how growth factor receptor-coupled signaling events may influence the function of Ran, or other nuclear GTP-binding proteins involved in nuclear signal transduction. In this study, we set out to identify possible GTP-binding activities in the nucleus which appear to be under growth factor control. To this end, we report the existence of an 18 kDa nuclear GTP-binding protein (herein referred to as p18) which responds to EGF, NGF, and heregulin addition to tissue culture cells with a marked increase in GTP-binding. This increased GTP-binding activity is also observed in cells arrested in the $G_1$/S phase of the cell cycle, but not in cells arrested in $G_0$ or M phase. We show that p18 exists as part of a high molecular weight complex within the nucleus, and that some component of this complex is necessary for GTP-GDP exchange upon p18. Furthermore, with the use of the temperature-sensitive tsBN2 cells, we place p18 downstream of RCC1 function, as the loss of RCC1 from these cells causes an increase in the GTP-binding activity of p18 as well as a mislocalization of p18 to the cytosol.

## EXPERIMENTAL PROCEDURES

*Cell culture and cell manipulations* - PC-12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and 10% horse serum. All other cell lines were maintained in DMEM (except for HeLa cells which where in RPMI) with 10% fetal bovine serum. The tsBN2 cell line (a generous gift from Dr. Claudio Basilico and Dr. Ian Macara, Vermont U.) is temperature-sensitive and is derived from the BHK21 cell line (15). Growth factor treatments were performed in PC-12 and HeLa cells. Prior to growth factor treatment, cells were switched to serum-free media for 40 hrs. Growth factors (NGF (Gibco-BRL), heregulin β1 (residues 177-244; a generous gift from Dr. Mark Sliwkowski, Genentech, Inc.), and EGF (Calbiochem) were then added to the serum-free media in concentrations and for times indicated in the "Results" section at 37°C. Following treatment, the growth factor-containing media was washed (2X) with Tris-buffered saline (TBS), and the cells were lysed. Cell cycle blocks were performed in HeLa cells. A $G_0$ block was achieved by switching to serum-free media for 22-24 hours. For $G_1$/S phase arrest, 2.5 mM thymidine was added to growth media for 22-24 hours. 80 ng/ml nocodazole was added to growth media for 22-24 hours to achieve arrest in M phase. After treatment, cells were collected, washed 2x with TBS, and lysed.

*Cell Fractionation and nuclear lysis* - Tissue culture cells were washed 2x on the plate with Tris-buffered saline and then lysed in a buffer containing Hank's buffer, pH 7.4, 0.3% NP-40, 1 mM sodium orthovanadate, 1 mM DTT, 100 mM phenylmethanesulfonyl fluoride (PMSF), and 10 μg/ml each of leupeptin and aprotinin. The lysate was then centrifuged for 15 minutes at 800 rpm at 4°C. The resulting supernatant was saved as the cytoplasmic fraction. The pellet was then washed with an equal volume of Hank's buffer with 0.2% TX-100, and centrifuged for 15 minutes at 800 rpm at 4°C. The resulting pellet was treated as the purified nuclear fraction. Nuclear lysis was performed in one of two

ways. For whole nuclear lysates, the nuclei were lysed in a buffer containing 50 mM Tris, pH 7.4, 1% TX-100, 400 mM KCl, 1 mM sodium orthovanadate, 1 mM DTT, and protease inhibitors as described above. The samples were incubated on ice for 30 minutes, microfuged, and the resulting supernatant was used as the whole nuclear fraction. To generate nuclear membrane, and nuclear soluble fractions, we followed the nuclear fractionation procedure described by Davis and Blobel (16) with some modification. The whole nuclear fraction was resuspended in 50 mM Tris-HCl pH 7.4, 10% (w/v) sucrose, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM $MgCl_2$, and protease inhibitors. DNase 1 (5 μg/ml) and RNase A (1 μg/ml) were added, and the nuclei were then incubated for 15 minutes at 37°C. The nuclei were underlayed with 30 % sucrose, and ultracentrifuged in a swinging bucket rotor for 10 minutes at 20,000 x g to generate a soluble fraction and a membrane fraction.

*Photoaffinity labeling of GTP-binding proteins* - Photoaffinity labeling of GTP-binding proteins with [$\alpha^{32}$P]GTP was performed as previously described (17). In brief, the UV crosslinking reaction was carried out in a crosslinking buffer that contained 50 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM DTT, 20% (v/v) glycerol, 100 mM NaCl, and 500 μM AMP-PNP. Samples (20 μl), prepared from cell fractionation procedures described above, were incubated for 10 minutes at room temperature with an equal volume of crosslinking buffer containing [$\alpha^{32}$P]GTP (2-3 μCi per sample) (New England Nuclear) in a 96 well tissue culture plate. The plate containing the samples was then placed on an ice bath and irradiated with UV light (254 nm) for 10 minutes. After irradiation, samples were mixed with 5x Laemmli buffer and boiled. SDS-PAGE was performed using 15% acrylamide gels. The gels were then typically Silver-stained, dried, and autoradiography was performed (typically overnight) using Kodak X-OMAT XAR-5 film at -80°C.

*Immunoprecipitation and immunoblotting* - A tsBN2 cell line which was stably transfected with $HA_1$-tagged RCC1 was a generous gift of Dr. Ian Macara. $HA_1$-tagged RCC1 was immunoprecipitated from nuclear lysates derived from these cells using the monoclonal antibody, 12CA5. In brief, whole nuclear lysate was prepared from asynchronously growing $HA_1$-tagged RCC1-tsBN2 cells. The nuclear lysate was then diluted with two parts 50 mM Tris-HCl pH 7.4, to one part lysate, and 750 µg of this diluted nuclear lysate was used for each immunoprecipitation. Either 25 µl of 12CA5 mAb or 25 µl of buffer was added to the lysates, and the samples were then allowed to incubate at 4°C for one hour. Following the first incubation, 40 µl of protein A Sepharose beads were added to each sample, and the samples were incubated for another hour at 4°C. The samples were then centrifuged, and the immunoprecipitated pellet was washed 4 times with 50 mM Tris-HCl pH 7.4, 100 mM KCl, 0.25% TX-100, 1 mM DTT, and 1 mM sodium orthovanadate. The resulting immunoprecipitated pellets were resuspended in 20 µl of UV-crosslinking buffer, and subjected to incubation with [$\alpha^{32}$P]GTP and UV-crosslinking as described above.

Western blotting for RCC1 was accomplished using a polyclonal RCC1 antibody which was a generous gift of Dr. Takeharu Nishimoto. For Western blotting analysis, proteins were transferred to PVDF membrane following SDS-PAGE. The PVDF membranes were blocked with 2% nonfat dry milk powder in TBS (pH 7.4) plus 0.1% Tween 20 for one hour at room temperature. After blocking, the membranes were incubated with RCC1 antibody for one hour at room temperature, washed with several changes of TBS-Tween, and incubated for one hour at room temperature with sheep-anti-rabbit horseradish peroxidase-conjugated antibody (Amersham) and washed. Immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

*Size determination of native p18* - Soluble nuclear lysate was prepared as described above from asynchronously growing PC-12 cells. Approximately 18 mg of lysate were loaded onto an FPLC, Superdex 200 Hiload 16/60 column (Pharmacia) in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM EDTA at a flow rate of 0.5 ml/min. Two ml fractions were collected and 30 μl of each fraction was assayed for p18 GTP-binding activity as described above, with the addition of 20 mM EDTA to compensate for the presence of 10 mM $MgCl_2$ in the gel filtration buffer.

*Gel filtration of nuclear lysates preloaded and UV-crosslinked with [$\alpha^{32}$P]GTP-* Soluble PC-12 nuclear lysate was prepared (approx. 15 mg), in a volume of 1.5 ml and made to a final EDTA concentration of 10 mM. The lysate was assayed in a 6-well tissue culture plate, with 500 μl lysate, 333 μl UV-crosslinking buffer, and 40 μCi [$\alpha^{32}$P]GTP per well. After UV-crosslinking, the 2.5 ml total crosslinked lysate was applied to a PD-10 gel filtration column (Pharmacia) to remove excess [$\alpha^{32}$P]GTP, and eluted with 3.5 ml superdex buffer. The UV-crosslinked lysate was then subjected to gel filtration as described above. Following fractionation, 30 μl of each fraction was subjected to SDS-PAGE and autoradiography.

## RESULTS

***p18 is nuclear protein which binds GTP in a growth factor dependent manner***- In order to investigate the possibility of the existence of a growth factor-sensitive, nuclear GTP-binding protein, HeLa cells were serum-starved and then challenged with a panel of growth factors. Following growth factor treatment for 15 minutes, the cells were then fractionated and the resulting cytoplasmic and nuclear lysates were assayed to determine if any protein showed a change in its ability to bind [$\alpha^{32}$P]GTP, as assayed by UV-crosslinking (see "Experimental Procedures"). As shown in Figure 1A (left panel), nuclear lysates from HeLa cells contained a GTP-binding activity that corresponded to a protein with an apparent $M_r$ of ~18 kDa. This activity was strongly stimulated by heregulin, and to a lesser extent by EGF. Thus far, a similar GTP-binding activity has been found in every cell line that we have tested including COS cells, human embryonic kidney cells (293), baby hamster kidney cells (BHK21), human epidermoid carcinoma (A431) cells, various mammary epithelial cells, and rat pheochromocytoma (PC-12) cells. In the case of PC-12 cells (shown in Figure 1A, right panel), the GTP-binding activity corresponding to an 18 kDa protein (from here on designated as p18) was strongly stimulated by NGF, as well as by heregulin and EGF. Figure 1B shows that the growth factor-stimulated binding of [$\alpha^{32}$P]GTP by p18 was dose-dependent. Half maximal binding of radiolabeled GTP occurred at ~10 nM heregulin and at less than 1 ng/ml NGF.

The original GTP-binding assays for p18 were performed after relatively short periods of growth factor treatment (i.e. 15 minutes). While this treatment was sufficient to detect [$\alpha^{32}$P]GTP-binding by p18, more complete time course experiments indicated that near maximal GTP binding required growth factor treatment for 1 hour. An example for PC-12 cells is shown in Figure 1C. In this experiment, serum-starved PC-12 cells were challenged with 100 ng/ml NGF for increasing time periods, up to 24 hours. The results show that peak GTP-binding to p18 occurred after approximately 1 hour of growth factor

addition and that the GTP-binding activity remained stimulated through the 24 hours of treatment. Thus, taken together, the data in Figures 1A-C identify p18 as a nuclear protein which binds GTP as part of a specific growth factor response and this response is increased and sustained with prolonged exposure of the cells to growth factor.

Cell fractionation experiments were performed to distinguish between the possibilities that p18 was associated with the nuclear envelope versus its presence in the soluble nuclear contents. To do this, purified nuclei were DNAase-digested to release soluble nuclear proteins and these proteins were then separated from nuclear membranes on a sucrose gradient. The nuclear membranes were detergent-solubilized and then both nuclear fractions, as well as the cytoplasmic S100 and P100 fractions, were analyzed for p18 as indicated by [$\alpha^{32}$P]GTP-binding activity. Figure 2 shows that the [$\alpha^{32}$P]GTP-binding activity corresponding to p18 was exclusively detected in the DNAase-digested, soluble nuclear fraction (lane 4) and was not detected in the cytosolic fraction (lane 1) nor in the microsomal fraction (lane 2) nor in nuclear membranes (lane 3). However, these results do not exclude the possibility that inactive p18 molecules are present in other cellular locations.

***Nucleotide binding to p18 is specific for guanine nucleotides***- While the binding of GTP to p18 appeared to be a specific event in response to growth factors, we felt that it was important to rule out the possibility that p18 was actually an ATP-binding protein. If this were the case, the incorporation of [$\alpha^{32}$P]GTP by p18 may simply have reflected a cross-reactivity. To examine this possibility, we compared the abilities of the non-hydrolyzable GTP-analog, GMP-PNP, and the non-hydrolyzable ATP-analog, AMP-PNP, to compete with [$\alpha^{32}$P]GTP for binding to p18. As shown in Figure 3A, the addition of increasing concentrations of GMP-PNP strongly inhibited the binding of [$\alpha^{32}$P]GTP to p18, with half maximal inhibition occurring at ~ 100 $\mu$M (lane 4). In contrast, AMP-PNP did not show measurable competition versus [$\alpha^{32}$P]GTP until levels of 10 mM were used.

A more direct comparison of nucleotide binding specificity is shown in Figure 3B. Here, nuclear lysates from either serum-starved or NGF-stimulated PC-12 cells were assayed for the binding of [$\alpha^{32}$P]GTP or [$\alpha^{32}$P]ATP to p18. While the nuclear lysates did contain proteins which bound to and were UV crosslinked to [$\alpha^{32}$P]ATP, these ATP-binding proteins did not correspond to p18. These results suggest that p18 has a significantly higher affinity for guanine nucleotides compared to adenine nucleotides.

An examination of the effects of divalent metals on the GTP-binding activity of p18 further suggests that p18 shares guanine nucleotide binding properties with members of the Ras superfamily. It previously has been shown that $Mg^{2+}$ blocks the dissociation of GDP from Ras-like proteins and thus inhibits the GDP-GTP exchange activity of these proteins (18-20). The addition of excess EDTA, by chelating $Mg^{2+}$, can then catalyze their guanine nucleotide exchange reaction (18,19). The same appears to be true for p18. As shown in Figure 3C, [$\alpha^{32}$P]GTP binding to p18 was strongly inhibited when the assay was performed in the presence of millimolar levels of $Mg^{2+}$, whereas the addition of EDTA stimulated the exchange of [$\alpha^{32}$P]GTP onto p18. In fact, EDTA treatment appears to circumvent the need for pretreating cells with growth factors in order to detect enhanced [$\alpha^{32}$P]GTP-binding to p18, in a manner analogous to what has been observed for Ras (20).

***The binding of GTP to p18 appears to be cell-cycle-dependent-*** The results presented in Figures 1-3 show that following serum starvation of cells, nuclear p18 binds GTP in response to growth factor treatment. We also have found that nuclear lysates from asynchronously growing cells possess an active p18. These findings suggest that the growth factor-dependent GTP-binding activity of p18 may be associated with a particular phase of the cell cycle. To investigate this possibility, HeLa cells were arrested in $G_0$ by serum starvation, in $G_1$/S by thymidine addition, and in M phase by nocodazole treatment (Figure 4). The cells were then fractionated into cytoplasmic and nuclear fractions (or

mitotic pellet in the case of M phase-arrested cells) and the resulting lysates were then assayed for the ability of p18 to bind [$\alpha^{32}$P]GTP. As expected based on our growth factor studies, we found that p18 did not bind GTP in cells arrested in $G_0$. p18 also did not bind GTP in either the cytoplasm or mitotic pellet of cells arrested in M phase. However, p18 did show strong GTP-binding activity in the nucleus of HeLa cells arrested in $G_1$/S. Thus the GTP-binding activity of p18 may undergo a cell-cycle-dependent regulation in a manner similar to the Ras-like nuclear GTP-binding protein Ran/TC4; specifically, Ran/TC4 is believed to bind GTP at the onset of S phase and to remain in an active GTP-bound form until DNA synthesis is complete and the cells exit S phase (11,12).

***p18 exists as part of a high molecular weight complex-*** It has been generally assumed that small proteins (Mr $\leq$ 60 kDa) are free to diffuse into and out of the nucleus through the nuclear pores and are retained in the nucleus only when they exist as part of a higher molecular weight complex. Since we have only detected p18 in the nucleus (at least as assayed by GTP-binding activity), we questioned whether p18 remains in the nucleus as part of a larger protein complex. To examine this possibility, nuclear lysates were subjected to gel filtration and the resulting fractions were assayed for [$\alpha^{32}$P]GTP-binding activity. The results presented in Figure 5A show that p18 GTP-binding activity was eluted from the gel filtration column as a high molecular weight complex, ranging from approximately 100-150 kDa. This suggests that active p18, in its native state, exists either as an oligomer (i.e. an octomer) or that it is present in a complex with other nuclear proteins.

It should be noted that we did not find [$\alpha^{32}$P]GTP-binding activity in fractions that corresponded to proteins with a molecular mass of 18-20 kDa. These results then indicate that either p18 is tightly associated in an oligomeric or multi-protein complex (such that there is little free p18) or that the monomeric form of p18 is not able to bind GTP. In order to investigate the later possibility, we performed a gel filtration experiment where the

nuclear lysate was subjected to loading and UV-crosslinking with [$\alpha^{32}$P]GTP prior to chromatography. Thus, even if p18 is required to be within a multi-protein complex (~150 kDa) in order to bind GTP, but then dissociates from this complex following GTP-binding, we should still be able to follow the elution of pre-labeled p18 from the gel filtration column by subjecting the fractions to SDS-PAGE and autoradiography. Figure 5B shows the elution pattern of proteins which were UV-crosslinked to [$\alpha^{32}$P]GTP prior to gel filtration. [$\alpha^{32}$P]GTP-labeled p18 can now be seen eluting both at 100-150 kDa and at 18 kDa. Taken together, the results from these experiments suggest that p18 exists in the nucleus both as a high molecular weight complex and as a monomeric species. Moreover, it appears that some component within the high molecular weight complex is responsible for the activation of p18, and once p18 binds GTP, it then is capable of dissociating from this complex.

***Interactions between p18 and the chromatin-associated protein, RCC1-*** RCC1 is a chromatin-associated, 45 kDa protein which exists in the nucleus in high molecular weight complexes (400-600 kDa or 150 kDa) (21) and functions as a guanine nucleotide exchange factor for the Ran GTP-binding protein (9). In *Saccharomyces cerevisiae*, the RCC1 homolog, PRP20, has been reported to associate not only with the Ran homolog, GSP1, but also with two additional GTP-binding proteins, one of which has a reported molecular weight of 17 kDa (22). Given the size distribution of RCC1 and the ability of the yeast homolog of RCC1 to bind to multiple nuclear GTP-binding proteins, we speculated that RCC1 might be a component of the p18 complex. Indeed, as RCC1 is a known guanine nucleotide-exchange factor, we wanted to examine whether the presence of RCC1 has any affect on the functional activity of p18. The temperature-sensitive cell line tsBN2 possesses a point mutation in the RCC1 gene such that the RCC1 protein becomes unstable and is degraded when these cells are shifted from the permissive temperature (33.5 °C) to the non-permissive temperature of 39. 5°C (23). Thus, this cell line allowed us to

determine whether the loss of RCC1 had any affect on p18 activity. Figure 6A shows the results obtained when the tsBN2 cells were grown at 33.5°C and then shifted to 39.5°C for 3 hours, 6 hours, and 24 hours. The effects on p18 that accompanied the loss of RCC1 were two-fold. First, the GTP-binding activity of p18 was significantly stimulated. Second, we found GTP-binding activity corresponding to p18 in the cytosol of tsBN2 cells that were temperature-shifted for 6 hours or 24 hours. Neither of these effects were observed in parental BHK21 (control) cells that were temperature-shifted in an identical manner (data not shown). These results then suggest that the presence of RCC1 can affect both the GTP-binding activity and the cellular location of p18.

As p18 increased its GTP-binding activity upon the loss of RCC1, it would appear that RCC1 does not act as a guanine nucleotide-exchange factor for p18. Nonetheless, the effect of the loss of RCC1 on p18 was dramatic, and we wanted to investigate whether the effect was due to a direct interaction between the two proteins. To test this possibility, $HA_1$-tagged RCC1 was immunoprecipitated from the BHK21-derived, temperature-sensitive cell line, tsBN2, which had been stably transfected with $HA_1$-RCC1. The immunoprecipitates were then assayed for the presence of p18 by UV crosslinking with [$\alpha^{32}$P]GTP. The results presented in Fig. 6B show that a small, but detectable, amount of p18 was present in the RCC1 immunoprecipitate but not in the control. Thus, these results suggested that p18 is capable of associating with RCC1 *in vivo*.

## DISCUSSION

GTP-binding proteins are known to function in the cell as tightly regulated molecular switches which play pivotal roles in a diversity of receptor-coupled signaling systems. The switching mechanism is accomplished by the exchange of GDP for GTP on the GTP-binding protein, thus rendering the protein active and allowing it to interact with target/effector molecules that mediate the transmission of a signal. The hydrolysis of GTP then serves to switch off the signaling pathway. Given the fundamental roles of GTP-binding proteins in signaling pathways occurring both at the plasma membrane and in the cytosol, it seems reasonable to envision a role for GTP-binding proteins in further downstream events such as transcriptional regulation, or cell-cycle control. Specifically, we sought the existence of a GTP-binding protein(s) responsible for mediating growth factor regulated nuclear events.

To this end, we have identified a nuclear, 18 kDa GTP-binding activity, designated p18, which is highly sensitive to the addition of growth factor to $G_0$ arrested cells. While much of our work characterizing the growth factor stimulation of p18 activity takes advantage of the sensitivity of PC-12 cells to NGF and heregulin, we have found p18 (or a related GTP-binding activity) in every cell line which we have investigated thus far. The ubiquitous nature of this protein suggests that it may be playing a fundamental role in growth factor signaling at the level of the nucleus.

Interestingly, though p18 responds immediately to growth factor addition, the GTP-binding activity does not become maximal until after approximately one hour of growth factor treatment. This activity is sustained through 24 hours of growth factor treatment, suggesting that the GTP-binding activity of p18 is not subject to down-regulation in this time frame. Furthermore, we found that p18 is able to bind GTP when cells are arrested in the $G_1$/S phase of the cell cycle, but not in $G_0$ or M phase, suggesting a cell-cycle dependence for this activity as well. We find it interesting that p18 can be

activated both by growth factor stimulation and by cell-cycle arrest, thus raising the possibility that the two activities might be coupled. For example, the growth factor activation of p18 may be necessary to initiate the cell to exit $G_0$, and once activated, p18 must then retain its activity to allow progression of the cell cycle.

While we have demonstrated that p18 activity is sensitive to growth factor addition to cells, we do not yet know the cascade of cytosolic events which culminate in the GTP-binding activity of p18. One well documented pathway from the plasma membrane to the nucleus involves the Ras-Raf-Mek-Erk signaling cascade. Since this pathway is central to mediating growth factor effects in the nucleus, it represents one possible mechanism for growth factor-stimulated p18 activation. However, our preliminary results suggest that the inhibition of Ras signaling (by use of a dominant-negative Ras mutant in PC-12 cells), does not effect the ability of p18 to respond to growth factor treatment. The involvement of other cell signaling pathways remains to be investigated, including protein kinase C and Jak/Stat. Additionally, we are investigating the involvement of stress-activated signaling pathways in p18 activation as we have found that p18 responds to UV-light exposure as well as to growth factor treatment (Wilson, unpublished data). Of particular interest is the pathway that begins with the activation of the PAK family (p21-activated serine/threonine kinases) by Cdc42 or Rac, and results in the stimulation of the stress-activated nuclear MAP kinases JNK1 and p38 (7,8). Moreover, there are a number of lines of evidence which indicate that signaling pathways stimulated by Rho-like GTP-binding proteins (e.g. Cdc42, Rac) are under growth factor control (5,6) making such pathways attractive candidates leading to p18 activation.

Though the cascade of events which translates growth factor binding at the cell surface into the activation of p18 is not clear, all indications thus far are that p18 is behaving similar to a Ras-like GTP-binding protein. That is, millimolar levels of $Mg^{2+}$ inhibit the guanine nucleotide exchange activity of p18 while EDTA addition can facilitate this exchange, as is the case for virtually all Ras-like proteins. This, in turn suggests that

growth factor signaling must activate a guanine nucleotide exchange factor (GEF) that stimulates the dissociation of GDP from p18. However, unlike the Ras proteins, we have not been able to load the monomeric pool of p18 with GTP (i.e. the low molecular weight pool that elutes from gel filtration columns, see Fig. 5B) by simple addition of EDTA. p18 must be associated with some component of the 100-150 kDa complex in order for the exchange of GDP for GTP to occur. In this regard, p18 behaves like some $\alpha$ subunits of trimeric G proteins (i.e., the $\alpha$ subunit of transducin can only bind GTP in the presence of an activated rhodopsin molecule (24)).

Given that p18 can bind GTP when it is part of a complex, but not as a monomer raises the possibility that the guanine nucleotide exchange factor for p18 might be part of this complex. Another way in which the GTP binding activity might be regulated is via the association/dissociation of a guanine nucleotide dissociation inhibitor (GDI). Since monomeric p18 cannot bind GTP, it is unlikely that a GDI could be the only mode of regulation for the GTP binding activity of p18, although a GDI may be working in concert with a GEF. It is interesting that p18 forms an apparently stable association with its putative nucleotide-exchange factor. Preliminary purification data indicates that the components of this complex can be copurified through at least three chromatography steps. A stable complex between a GTP-binding protein and a GEF is not without precedent. Indeed, the nuclear GTP-binding protein, Ran, was copurified with RCC1 (25,26), a protein which was later shown to have exchange activity on Ran. (9)

An obviously important question concerns the identity of the guanine nucleotide exchange factor for p18. RCC1 was one obvious candidate since it has already been identified as a GEF for Ran, and because a 17 kDa nuclear GTP-binding protein has been found to associate the *S. cerevisiae* RCC1 homolog, PRP20 (21). Furthermore, it is plausible to envision RCC1 (in conjunction with p18) as a downstream component of a growth factor-mediated signaling event given that an *S. cerevisiae* RCC1 homolog, SRM1, was identified as a suppresser of a deletion of the **a**-factor receptor gene, thereby placing

SRM1 (downstream) in a receptor-mediated signaling event in yeast (27). Using the temperature-sensitive cell line, tsBN2, to investigate whether RCC1 could be a GEF for p18, we found that the loss of endogenous RCC1 did influence the GTP-binding ability of p18. However, instead of causing a concomitant decrease in GTP-binding activity as would be expected for a decrease in the amount of a GEF, we instead found that the loss of RCC1 actually caused an increase in the ability of p18 to bind GTP. Thus, RCC1 cannot be the GEF for p18. An additional and potentially interesting finding was that p18 GTP-binding activity could be detected in the cytosol when RCC1 levels were decreased. Both of these observations, together with the finding that p18 co-immunoprecipitates with RCC1, are consistent with a direct interaction between p18 and RCC1. RCC1 may in fact be acting as a GDI for p18 such that when it is no longer available to bind to p18, p18 is free to be activated by its exchange factor. The loss of RCC1 could also be responsible for generating a lower molecular weight form of p18 which is free to diffuse into the cytosol. This would be similar to the translocalization of Ran from the nucleus into the cytosol upon temperature shifting tsBN2 cells (10,28). If RCC1 is acting as a GDI for p18, this would additionally offer an explanation as to why only a small amount of p18 GTP-binding activity is capable of co-immunoprecipitating with RCC1. In this case, there may not be a positive relationship between the amount of protein associating with RCC1 and the amount of p18 GTP-binding activity observed.

However, it is important to note that shifting tsBN2 cells to their restrictive temperature causes duel cell-cycle defects. Cells shifted in $G_1$ phase arrest at the $G_1/S$ transition, while cells which are in S phase when the shift occurs are subject to premature chromosome condensation and entry into mitosis (14). As we have shown with the thymidine arrest, the GTP-binding activity of p18 is dramatically increased when cells are blocked in $G_1/S$ phase compared to $G_0$ or M phase. Therefore, the increased GTP-binding of p18 at the nonpermissive temperature may be reflecting the $G_1/S$ block, independent of any direct association between p18 and RCC1. Similarly, the displacement of p18 GTP

binding activity into the cytosol when the tsBN2 cells are shifted to their restrictive temperature may be explained by a nuclear transport defect. It has been demonstrated that the loss of RCC1 in 39.5°C tsBN2 cells disrupts the efficiency of nuclear transport (28). This is most likely due to the inability of RCC1 to function on Ran, which in addition to its other properties, is also known to be a requirement for protein import into the nucleus (29,30). Therefore, the accumulation of p18 and/or its exchange factor in the cytosol of tsBN2 cells at the restrictive temperature could be attributed to a general nuclear transport defect rather than to a physical association between p18 and RCC1. Distinguishing between these possibilities will most likely be dependent on developing methods for detecting p18 independent of its GTP-binding activity.

In conclusion, we have identified a nuclear GTP-binding protein whose specific, GTP-binding activity is stimulated both by growth factor addition and cell-cycle progression. In addition, we have shown an association between p18 and RCC1, a protein involved in diverse nuclear functions including cell-cycle checkpoint control. The coincidence of both a growth factor and a cell-cycle input into the regulation of this GTP-binding protein suggests that p18 may play a role in governing the $G_1$ checkpoint of the cell-cycle. In order to further our understanding of p18, the interplay between p18 and RCC1, and the roles of both growth factor signaling and the cell-cycle in this interaction, future studies will be directed toward the purification and molecular cloning of this 18 kDa nuclear GTP-binding protein.

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## FIGURE LEGENDS

FIG. 1. **Growth factor activation of an 18 kDa nuclear GTP-binding protein in HeLa cells and PC-12 cells.** A) HeLa cells (*lanes 1-6*) or PC-12 cells (*lanes 7-14*) were serum-starved (*control lanes 1,4,7,and11*) and then treated with 100 ng/ml EGF (*lanes 2,5,9,13*), 30 nM heregulin (*lanes 3,6,8,12*), or 100 ng/ml NGF (*lanes 10,14*) for 15 minutes at 37°C. The cells were then lysed and separated into cytoplasmic (*lanes 1-3, 7-10*), and whole nuclear (*lanes 4-6, 11-14*) fractions and assayed for GTP-binding using 45 μg of HeLa lysate or 80 μg of PC-12 lysate. The samples were incubated for 10 minutes at room temperature with 20 μl crosslinking buffer and 3 μCi [$\alpha^{32}$P]GTP followed by exposure to UV light for 10 minutes on ice. The samples were then separated by 15% SDS-PAGE, and the resulting gel was dried and exposed to X-ray film for 11 hrs to 3 days. B) A dose-response experiment was performed using the addition of either heregulin (*lanes 1-5*) or NGF (*lanes 6-9*) to serum-starved PC-12 cells (*control, lane 2*) for 30 minutes at 37°C. 50 μg of whole nuclear lysate generated from each dose treatment was assayed for GTP-binding by UV-crosslinking with [$\alpha^{32}$P]GTP, and was then separated by 15% SDS-PAGE. The resulting gel was dried and exposed to X-ray film for 5-15 hrs. C) A time course of 100 ng/ml NGF treatment was performed in serum-starved PC-12 cells (*control, lane 1*) with NGF addition for 7.5 minutes (*lane 2*), 15 minutes (*lane 3*), 30 minutes (*lane 4*), 60 minutes (*lane 5*), or 24 hrs (*lane 6*). 50 μg of resulting whole nuclear lysate was assayed for binding to [$\alpha^{32}$P]GTP by UV-crosslinking, followed by 15% SDS-PAGE and autoradiography overnight.

FIG. 2. **The GTP-binding activity of p18 is localized to the soluble nuclear contents.** 23 g of PC-12 cells were fractionated into a cytosolic, S100 fraction (*lane 1*), a microsomal, P100 fraction (*lane 2*), a nuclear membrane fraction (*lane 3*), and a DNase I-digested, soluble nuclear fraction (lane 4). The p100 fraction was extracted in Tris-HCl pH

7.4, 1% TX-100, 400 mM KCl, and 1 mM DTT, and the nuclear membrane fraction was extracted with Tris-HCl pH 7.4, 1% TX-100, 800 mM KCl and 1 mM DTT. 45 μg of each fraction was assayed in the presence of 20 μl crosslinking buffer for GTP-binding by UV-crosslinking to [$\alpha^{32}$P]GTP. The samples were then separated by 15% SDS-PAGE, and the resulting gel was then dried and exposed to X-ray film overnight.

FIG. 3. **The nucleotide binding activity of p18 is specific for guanine nucleotides.** A) 50 μg of PC-12, DNase I-digested, soluble nuclear lysate was subjected to crosslinking to [$\alpha^{32}$P]GTP in the presence of increasing concentrations of cold AMP-PNP [ 0 μM (*lane 1*), 5.0 μM (*lane* 2), 10 μM (*lane 3*), 50 μM (*lane 4*), 100 μM (*lane 5*), 500 μM (*lane 6*), 1.0 mM (*lane 7*), 5.0 mM (*lane 8*), and 10 mM (*lane 9*)] and in the presence of cold GMP-PNP [ 0 μM (*lane 10*), 5.0 μM (*lane 11*), 10 μM (*lane 12*), 50 μM (*lane 13*), 100 μM (*lane 14*), 500 μM (*lane 15*), 1.0 mM (*lane 16*), 5.0 mM (*lane 17*), and 10 mM (*lane 18*)]. Following UV-crosslinking, samples were separated by 15% SDS-PAGE, and the resulting gel was dried and exposed to X-ray film overnight. B) Serum starved PC-12 cells were treated with 100 ng/ml NGF for 15 minutes at 37°C. 50 μg of whole nuclear extract from serum-starved cells (*lanes 1,3*) or NGF-treated cells (*lanes 2,4*) was incubated and crosslinked in the presence of [$\alpha^{32}$P]GTP (*lanes 1,2*) or [$\alpha^{32}$P]ATP (lanes 3,4). Samples were separated by 15 % SDS-PAGE, and the resulting gel was dried and exposed to X-ray film overnight. C) 45 μg of whole nuclear lysate from PC-12 cells was UV-crosslinked with [$\alpha^{32}$P]GTP (*lane 1*) in the presence of 100 μM cold GTP (*lane* 2), 100 mM $MgCl_2$ (*lane 3*), or 10 mM EDTA (*lane 4*). Following crosslinking, samples were subjected to 15% SDS-PAGE and autoradiography.

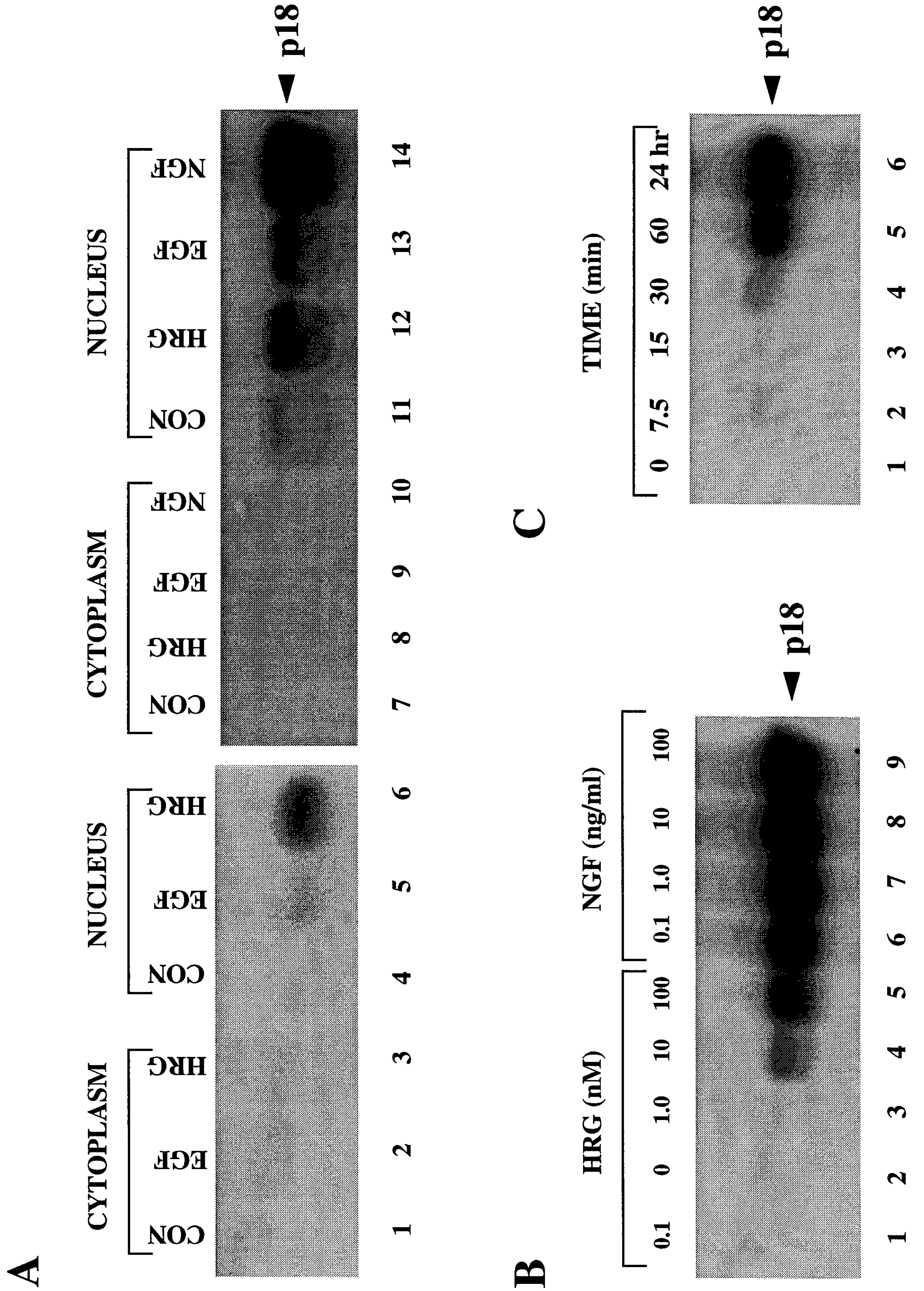
FIG. 4. **p18 is active to bind GTP in cells arrested in $G_1$/S phase of the cell cycle.** HeLa cells were arrested in $G_0$ by serum starvation (*lanes 1,4*), in $G_1$/S by 2.5 mM thymidine addition (*lanes 2,5*), and in M phase with 80 ng/ml nocodazole (*lanes 3,6*).

Cells were then separated into cytoplasmic (*lanes 1,2,3*), and whole nuclear fractions (or mitotic pellet for M phase arrest) (*lanes 4,5,6*), and 50 μg of each lysate was assayed for [$\alpha^{32}$P]GTP-binding activity by UV-crosslinking followed by 15% SDS-PAGE and autoradiography.

FIG. 5. **p18 exists in the nucleus both as part of a high molecular weight complex and as a monomer**. A) PC-12 DNase I-digested, soluble nuclear extract (18 mg) was fractionated, using a Superdex 200 column, into 2 ml fractions. 30 μl of each fraction was then subjected to UV-crosslinking with [$\alpha^{32}$P]GTP in the presence of 10 μl crosslinking buffer and 20 mM EDTA, followed by 15% SDS-PAGE and autoradiography. B) PC-12, DNase I-digested, soluble nuclear extract (15 mg) was subjected to UV-crosslinking with [$\alpha^{32}$P]GTP in the presence of 1.0 ml crosslinking buffer and 10 mM EDTA. Following crosslinking, unbound [$\alpha^{32}$P]GTP was removed from the lysate using a PD10 column. The crosslinked lysate was then applied to a Superdex 200 column, and 2 ml fractions were collected. 30 μl of each fraction were then separated by 15 % SDS-PAGE. The resulting gel was dried and exposed to X-ray film for 8 days to visualize the elution of [$\alpha^{32}$P]GTP-crosslinked proteins. Gel filtration standards are from Biorad.

FIG. 6. **Loss of cellular RCC1 causes an increase in p18 GTP-binding activity and a translocation of active p18 into the cytosol**. A) tsBN2 cells grown at 33.5°C (*lanes 2,6*) were shifted to 39.5°C for 3 hrs (*lanes 2,6*), 6 hrs (*lanes 3,7*) or 24 hrs (*lanes 4,8*) and then fractionated into cytoplasmic and whole nuclear lysates. 50 μg of each lysate were then separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted for RCC1 (*top panel*) or assayed for GTP-binding activity by UV-crosslinking with [$\alpha^{32}$P]GTP (*bottom panel*). The crosslinked samples were then separated by 15% SDS-PAGE, and the resulting gel was dried and exposed to X-ray film

overnight. B) $HA_1$-RCC1 was immunoprecipitated from a stably transfected tsBN2 cell line using the specific 12CA5 monoclonal antibody with protein A sepharose (*lane 3*) or protein A sepharose alone (*lane 2*). 30 µl of whole nuclear lysate (*lane 1*) and the immunoprecipitated pellets were then subjected to UV-crosslinking with [$\alpha^{32}$P]GTP. Following crosslinking, samples were separated by 15% SDS-PAGE, and the resulting gel was dried and exposed to X-ray film overnight.


A
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1 2 3 4 5 6 7 8 9 10 11 12 13 14
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p18
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Figure 1

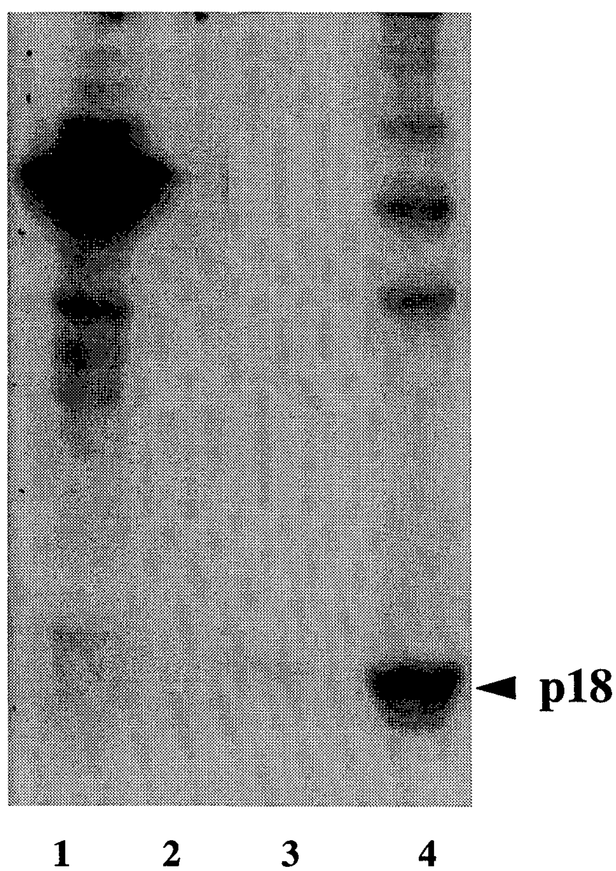
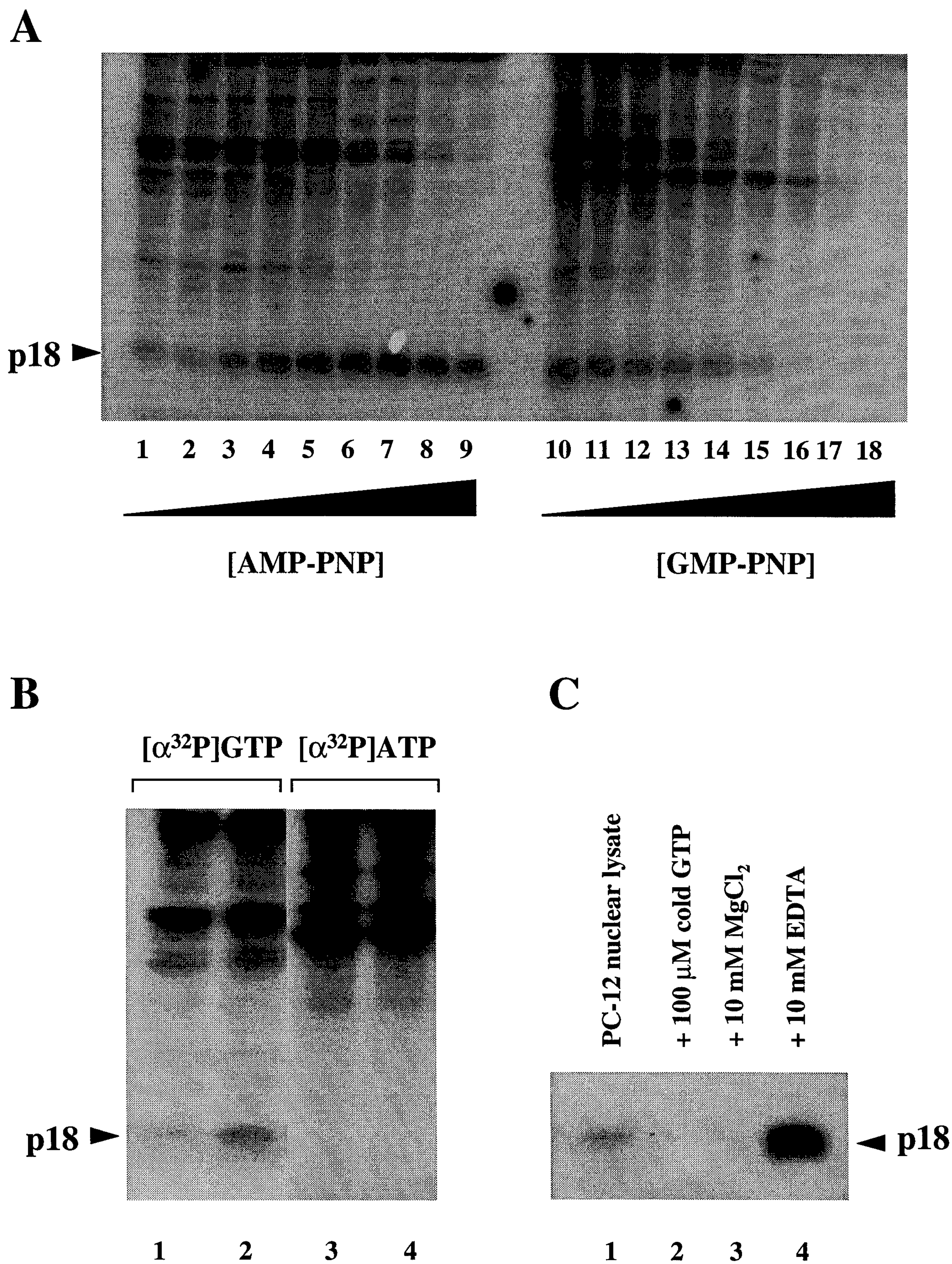



Figure 2



A
p18
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18
[AMP-PNP]
[GMP-PNP]
B
[α32P]GTP
[α32P]ATP
p18
1 2 3 4
C
PC-12 nuclear lysate
+ 100 μM cold GTP
+ 10 mM MgCl2
+ 10 mM EDTA
p18
1 2 3 4


Figure 3

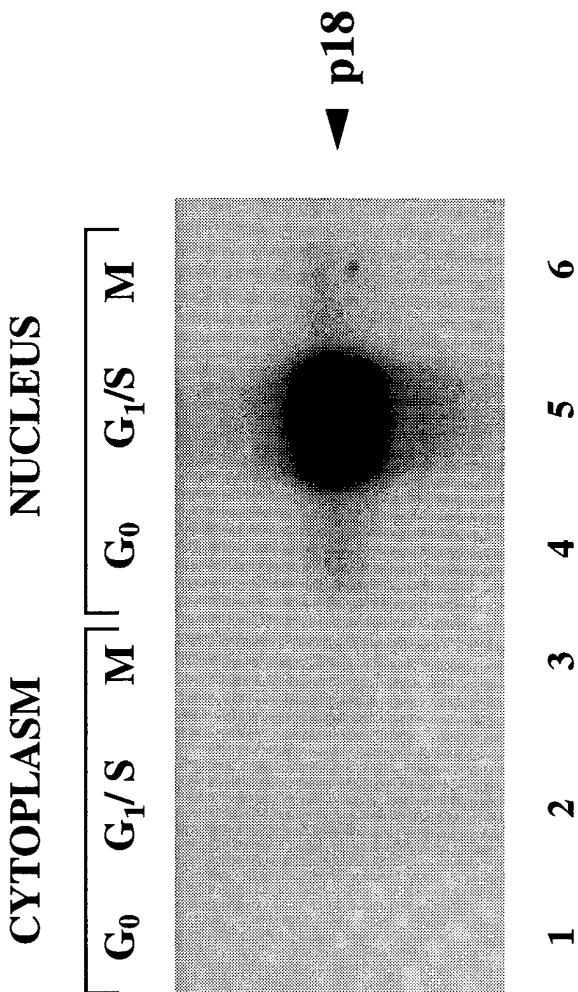



Figure 4

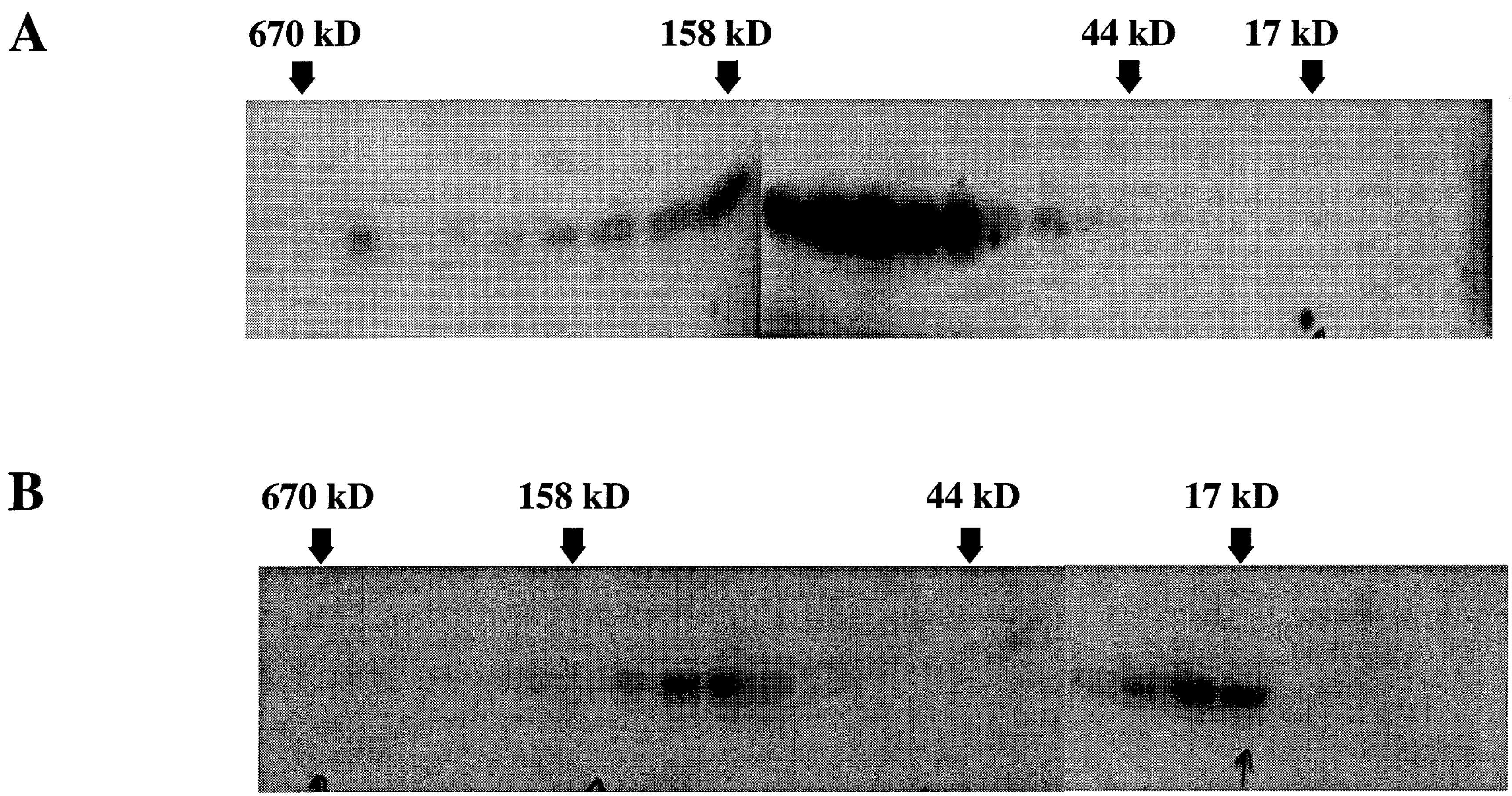
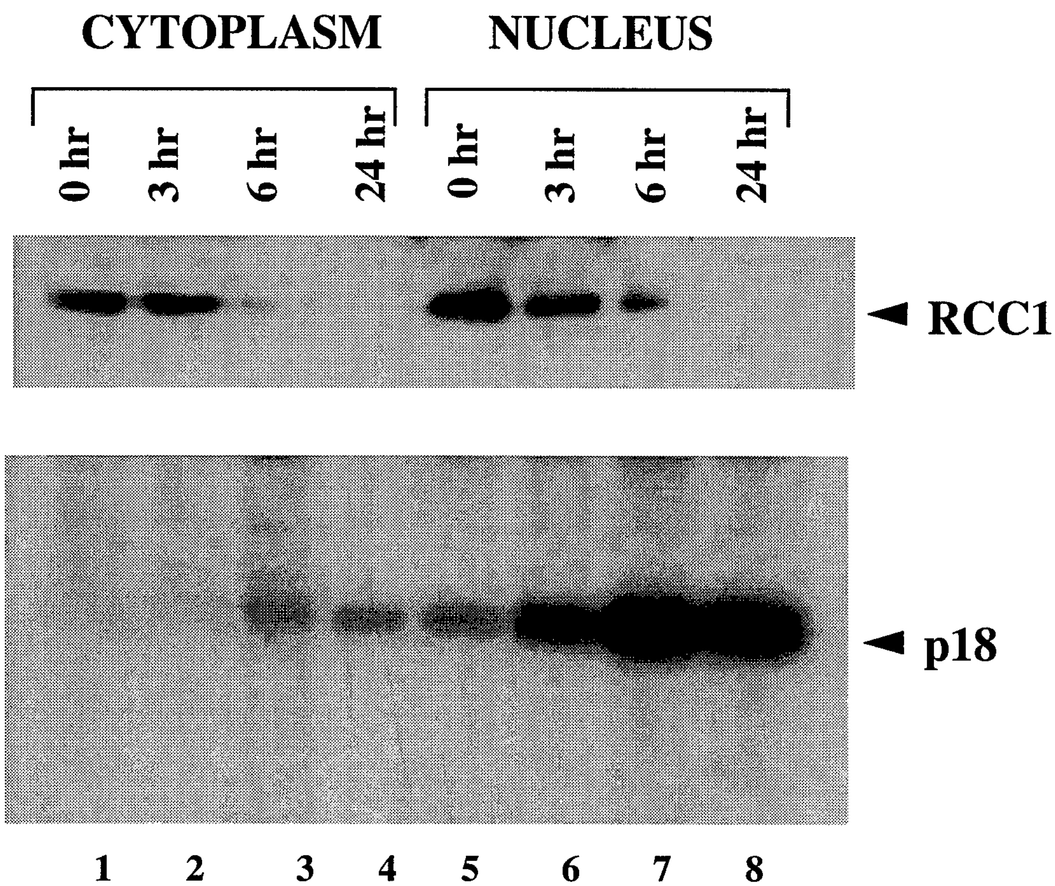



Figure 5

A



CYTOPLASM
NUCLEUS
0 hr
3 hr
6 hr
24 hr
0 hr
3 hr
6 hr
24 hr
RCC1
p18
1
2
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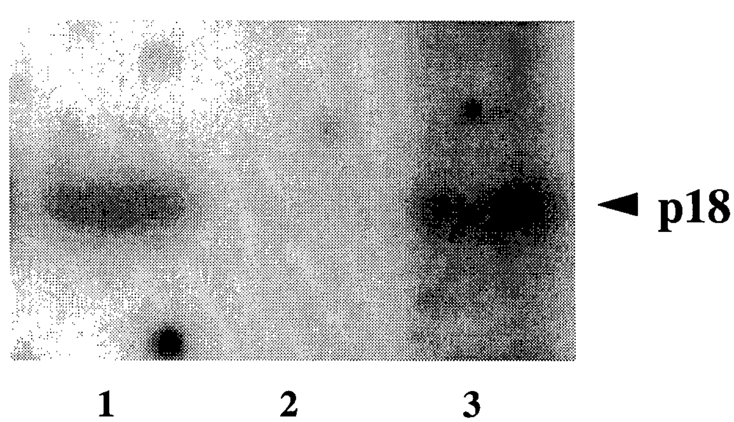
4
5
6
7
8


B



p18
1
2
3


Figure 6

# The Pleckstrin Homology Domain Mediates Transformation by Oncogenic Dbl Through Specific Intracellular Targeting

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## ABSTRACT

The Pleckstrin homology (PH) domain is an ~100 amino acid structural motif found in many cellular signaling molecules including the Dbl oncoprotein and related, putative guanine nucleotide exchange factors (GEFs). Here we have examined the role of the Dbl PH (dPH) domain in the activities of oncogenic Dbl. We report that the dPH domain is not involved in the interaction of Dbl with small GTP-binding proteins and is incapable of transforming NIH 3T3 fibroblasts. On the other hand, co-expression of the dPH domain with oncogenic Dbl inhibits Dbl-induced transformation. A deletion mutant of Dbl that lacks a significant portion of the PH domain retains full GEF activity, but is completely inactive in transformation assays. Replacement of the PH domain by the membrane-targeting sequence of Ras is not sufficient for the recovery of transforming activity. However, subcellular fractionations of Dbl and Dbl mutants revealed that the PH domain is necessary and sufficient for the association of Dbl with the Triton X-100 insoluble cytoskeletal components. Thus, our results suggest that the dPH domain mediates cellular transformation by targeting the Dbl protein to specific cytoskeletal locations to activate Rho-type small GTP-binding proteins.

## INTRODUCTION

The cytoskeletal-associated Dbl oncoprotein transforms NIH 3T3 cells (1) by activation of signaling pathways involving Rho-type GTP-binding proteins (2). Proto-Dbl is an 115 kDa cytoskeletal-associated protein that is found in brain, adrenal glands, and gonads (1). Oncogenic activation occurs as an outcome of an amino-terminal truncation of proto-Dbl, where a recombination event fuses about 10 kDa of an unidentified human gene product (from chromosome 3) on to the carboxyl terminal half of Dbl (residues 498-925). The oncogenic Dbl protein contains at least two putative signaling motifs. The first is a region of 176 amino acids (residues 498 to 674) that was originally found to share significant homology with the *Saccharomyces cerevisiae* cell-division-cycle protein, Cdc24, and the breakpoint cluster region protein, Bcr[1] (3). This region, referred to as the Dbl-homology (DH) domain, has been shown to be essential both for the transformation activity of oncogenic Dbl and for its ability to act as a GEF by stimulating the guanine nucleotide exchange activity of Cdc42Hs (4,5). The second putative signaling motif is the Pleckstrin-homology (PH) domain (6,7) and includes residues 703-812. Although PH domains appear to be relatively poorly conserved, both Nmr and X-ray crystallographic studies of the PH domains of Pleckstrin, Dynamin, and Spectrin indicate that they adopt a common 3-dimensional structural motif (8-11).

Over the past few years, a growing family of oncogene products and other growth regulatory proteins have been shown to contain a DH domain in tandem with a PH domain. In addition to Cdc24 and Bcr, these include the Vav, Ost, Ect-2, Lbc, Lfc, and Dbs oncoproteins (12-17) and the activators of the Ras proteins, Sos (18), and Ras-GRF (19). All indications from previous studies are that the DH domain will form a binding site and in many cases contain GEF activity for Rho-like GTP-binding proteins (8,9,13,14,20,21,22). However, less is known about the roles of the PH domains. In the present study, we have used the Dbl oncoprotein as a model to examine the role of the PH domain in cellular transformation and GEF activity.

## EXPERIMENTAL PROCEDURES

**cDNA Transfection Studies-** Transfection assays were done on duplicate cultures by adding 0.001 μg, 0.01 μg, 0.1 μg, and 1 μg of DNA to the recipient NIH 3T3 cells using the $Ca^{2+}$-phosphate precipitation method (3). Foci (focus forming units, ffu) were scored 14 days after transfection, and the results were calculated as number of foci per picomole of DNA. The results listed in Figure 1 and shown in Figure 2C are the mean values of three transfection assays. Growth in soft agar was examined as described by Ron et al. (3).

**Cellular Fractionation Studies-** Control NIH 3T3 and different NIH 3T3 transfectants were lysed and fractionated into cytosolic (S), Triton X-100-solubilized membrane fractions (T), and Triton X-100 insoluble fractions (I) as described by Graziani et al. (1). Cells were labeled with [$^{35}$S]-Methionine and [$^{35}$S]-Cysteine for three hours at 37°C. Specific Dbl products were detected by immunoprecipitation using anti-Dbl-2 antibodies (3), electrophoresed through a 12% polyacrylamide gel and autoradiographed. For the detection of the PH domains (e.g. Figures 2B and 4D, below), cells were immunoprecipitated with anti-Flag M5 antibodies and electrophoresed through a 15% polyacrylamide gel followed by immunoblotting with anti-Flag M5 antibodies.

**Measurements of GDP Dissociation from Cdc42Hs-**. The [$^3$H]GDP dissociation assays were carried out as previously described (4,5). In Figure 3A, the amounts of GST-Dbl and GST-DH (see Figure 1) purified from Sf9 insect cells were estimated by Coomassie-blue staining after SDS-PAGE. ~200 nM of GST-Dbl or GST-DH were incubated with 1 μg RhoA protein preloaded with [$^3$H]GDP in 100 μl of reaction buffer at room temperature, and 16 μl aliquots were diluted into 5 ml of ice-cold termination buffer (20 mM TrisHCl, pH 7.4, 10 mM $MgCl_2$, and 100 mM NaCl) at various time points. In Figure 3B, 1 μg of [$^3$H]GDP-bound RhoA was incubated with 2 μM GST, 2 μM GST-PH (a fusion protein containing GST and the Pleckstrin-homology domain

from the Dbl protein), 300 μM GST-Dbl, or 300 μM GST-Dbl and 2 μM GST-PH in a 100 μl reaction mixture.

## RESULTS AND DISCUSSION

To investigate the role of the PH domain in cellular transformation mediated by the oncogenic Dbl protein, we analyzed several Dbl mutants for transforming activity in NIH 3T3 cells (Figure 1). We found that while the transforming capability of a deletion mutant containing primarily the DH and PH domains (designated as pMA4 in Figure 1) was similar to that of the Dbl oncogene product, neither the DH domain nor the PH domain (pdDH and pdPH, respectively) alone showed any detectable effects on the growth of 3T3 fibroblasts. However, when Dbl and pdPH were co-expressed in NIH 3T3 cells (dbl+pFlag/PHdbl, Figure 2C), we observed a significant reduction of the transforming activity by Dbl. Co-expression of Dbl with a Flag-tagged PH domain of the Dbl-related Vav oncoprotein (12), on the other hand, showed effects comparable to those obtained with the pFlag/neo vector control (Figure 2C), even though it appeared to be more highly expressed (Figure 2B, lane 2) than the PH domain of Dbl (Figure 2B, lane 1). The level of expression of the Dbl oncoprotein was essentially equivalent in all cases (Figure 2A).

To further confirm the selective inhibition of Dbl-induced transformation by the dPH domain, we used a second mammalian expression vector, pKH3 (23), to express the PH domain from either Dbl, Vav, or from the related yeast Cdc24 protein (20), together with the Dbl oncogene product. As shown in Figure 2C, the expression of the dPH domain inhibited the focus-forming activity of oncogenic Dbl by ~40%, whereas co-expression of Dbl with the PH domain of Vav (pKH3/PHvav) had little effect. Mass populations of these transfected cells also were examined for their ability to display anchorage-independent growth. We observed that cells co-expressing Dbl and the dPH domain lost the ability to grow in soft agar (Figure 2D). In some cases, as shown in Figure 2D, we found that expression of a Flag-tagged PH domain of Vav caused some inhibition of Dbl-induced growth in soft agar, suggesting that the Vav PH domain (perhaps when expressed at sufficient levels) was capable of competing with the PH domain of Dbl for a cellular target. However, it is likely that the Vav PH domain is a weak competitor, since we often observed no detectable effects with either the Flag-tagged protein or when expressing the PH

domain of Vav from the pKH3 vector. We also have found no detectable effects on Dbl transformation when expressing the PH domain from Cdc24. Mass cultures of Dbl-transfectants expressing the dPH domain also displayed a less transformed phenotype compared to Dbl-transfectants alone, or compared to cells co-expressing Dbl and the PH domain of Vav (data not shown). Taken together, these results suggest that the PH domain of Dbl behaves as a selective antagonist of Dbl-induced transformation, possibly by binding to a saturable and specific ligand in cells.

Previously we have shown that the DH domain alone is sufficient for the GEF activity for Cdc42Hs (5). Since oncogenic Dbl also stimulates the guanine nucleotide exchange activity of Rho, we examined whether the Dbl domain is sufficient for stimulating the activation of Rho. To do this, we compared Rho-GEF activities of approximately equal amounts (~200 nM) of insect cell-expressed, purified GST-Dbl and GST-DH domain. No significant differences were observed for the abilities of the GST-Dbl and GST-DH to stimulate [$^3$H]GDP dissociation from RhoA (Figure 3A). These results suggest that the PH domain does not contribute to the GEF function of Dbl. This is further reinforced by the results in Figure 3B which show that the addition of excess *E. coli* recombinant PH domain to GEF assay mixtures containing [$^3$H]GDP-bound RhoA and recombinant GST-Dbl has no detectable effect on the time-course of GST-Dbl-stimulated [$^3$H]GDP dissociation from RhoA. The GST-PH domain, alone, also shows no ability to stimulate [$^3$H]GDP dissociation from RhoA (compared to GST alone). Similar results were also obtained with [$^3$H]GDP-bound Cdc42Hs (data not shown). Thus, the dPH domain is not involved in the interactions of Dbl with RhoA and Cdc42Hs or in the direct regulation of the GEF catalytic activity of the DH domain.

The membrane association of βARK and Spectrin has been attributed to their PH domains (24,25). The PH domains of βARK, BTK, PLCγ, IRS-1, and Ras-GRF have been shown to bind to plasma membrane-associated βγ subunits of the heterotrimeric G-proteins (26,27), and they all behave as antagonists of Gβγ-mediated signaling (28). Recent evidence also suggests that PH domains from many signaling molecules including βARK and Ras-GAP can bind to specific

phospholipids, namely PIP2 (29). These findings raised the possibility that the PH domain mediates the membrane targeting of oncogenic Dbl. It has been shown that the introduction of a membrane-targeting sequence into the Ras GEFs, Cdc25 and Sos (30,31), was sufficient to activate Ras, and more recently, that the addition of a membrane-targeting sequence in place of the PH domain of the Lfc oncoprotein was able to restore full transformation activity (32). Thus, we examined whether the substitution of the dPH domain with a membrane-targeting sequence enabled the DH domain of Dbl to induce transformation. A chimeric molecule containing the DH domain (residues 498-756) fused to the Ras membrane-targeting farnesylation signal sequence (designated pdHF in Figure 1) was constructed and assayed for focus-forming activity in NIH 3T3 cells. This chimera was expressed at a comparable level to oncogenic Dbl and a percentage (10-20%) of the total chimeric molecules were targeted to the membrane surface [i.e. the Triton X-100 solubilized fraction (T) in Figure 4A]. However, this did not restore transforming activity to the DH domain (Figure 1). Although, one possible explanation is that the amount of the chimera expressed at the membrane surface was not sufficient to stimulate a transforming signal, this does not seem likely based on what we have observed regarding the range of expression of oncogenic Dbl that will yield cellular transformation (34).

We have previously reported that significant portions of both proto- and oncogenic Dbl are localized to the Triton X-100 insoluble fractions of transfected NIH 3T3 cells, suggesting an association with the cytoskeletal matrix (1). To address the possible role of the dPH domain in mediating this pattern of localization for the Dbl protein, stable transfectants of Dbl and Dbl deletion mutants (Figure 1) were subjected to subcellular fractionation. The crude membrane fractions (P100) of the cells were solubilized either by 1% Triton X-100 or by treatment with 0.1% SDS and 0.25% sodium deoxycholate (DOC). Anti-Dbl immunoprecipitates revealed that a percentage of both the intact oncogenic Dbl protein and a truncation mutant pMA4 associated with the Triton X-100 insoluble fractions of cells (designated by I in Figures 4B and 4C). The amounts of oncogenic Dbl and pMA4 that were present in the Triton X-100 insoluble fraction typically varied between 50% and 70% of the total detectable protein, although in some cases (Figure 4B)

the percentage of oncogenic Dbl in this fraction was less than 50%. However, the DH domain of Dbl, which lacks transforming ability, was localized exclusively to the cytosolic fraction (designated S in Figure 4C). When cells expressing the Flag-tagged PH domains were subjected to similar fractionation, the PH domains were found associated with the Triton X-100 insoluble fractions (Figure 4D). These results suggest that the dPH domain is directly responsible for the association of oncogenic Dbl with the Triton X-100 insoluble cytoskeletal fraction, and thus may serve to target the catalytic DH domain to the cytoskeleton.

We have previously reported that the DH domain is responsible for Dbl GEF function and is required for Dbl transforming activity (3,5). Here, we demonstrate that while the dPH domain does not seem to be involved in the interactions of Rho-type small GTP-binding proteins with Dbl, it is essential for Dbl transforming activity. Thus, our present findings establish that both the DH and PH domains are required for the cellular function of Dbl. Indeed, the minimum structural unit (pMA4) of oncogenic Dbl confering complete transforming activity just encompasses the DH domain and the PH domain. The finding that plasma membrane-targeting of Dbl is not sufficient to confer transforming activity, coupled with the requirement of the dPH domain as the necessary and sufficient element for association of the Dbl protein with the Triton X-100 insoluble component, suggests that the function of the PH domain resides in its ability to target the catalytic DH domain to the cytoskeletal matrix. Whether this targeting function holds for other members of Dbl-related GEF family proteins remains to be seen. However, based on the observation that the PH domains of Dbl-related molecules Vav and Cdc24 do not act effectively as inhibitors of Dbl-induced transformation, it is an attractive possibility that different members of the family of Dbl-related proteins may be targeted by their PH domains to distinct cellular locations to activate various Rho-type GTP-binding proteins, in response to different extracellular stimuli such as EGF, PDGF, LPA, and Bradykinin. This may also explain the finding that substitution of a membrane-targeting (i.e. Ras-farnesylation) sequence for the PH domain of Lfc restored its transformation capability (32), whereas this substitution did not restore transforming activity to a Dbl protein that just contains the DH domain. It may be that Lfc needs to be targeted to the plasma

membrane to optimally couple to other protein components in its signaling pathway while Dbl needs to be targeted to a cytoskeletal location.

The identity of the ligand(s) that binds to the PH domain of oncogenic Dbl will represent an important focus of future studies. It seems likely, that given the hypervariable nature of the putative ligand-binding cleft in the PH domains that have thus far been identified (33), a complex diversity of ligands may exist which are responsible for mediating the actions of various PH domain-containing signaling molecules, including Dbl and related regulatory molecules of small GTP-binding proteins.

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[1] The abbreviations used are: Bcr, break point cluster region protein; GAPs, GTPase-activating proteins for low molecular mass GTP-binding proteins; GRF, guanine nucleotide-releasing factor; GEFs, guanine nucleotide-exchange factor for low molecular mass GTP-binding proteins; GST, glutathione-S-transferase; GTP$\gamma$S, guanosine 5'-3-O-(thio)triphosphate.

## FIGURE LEGENDS

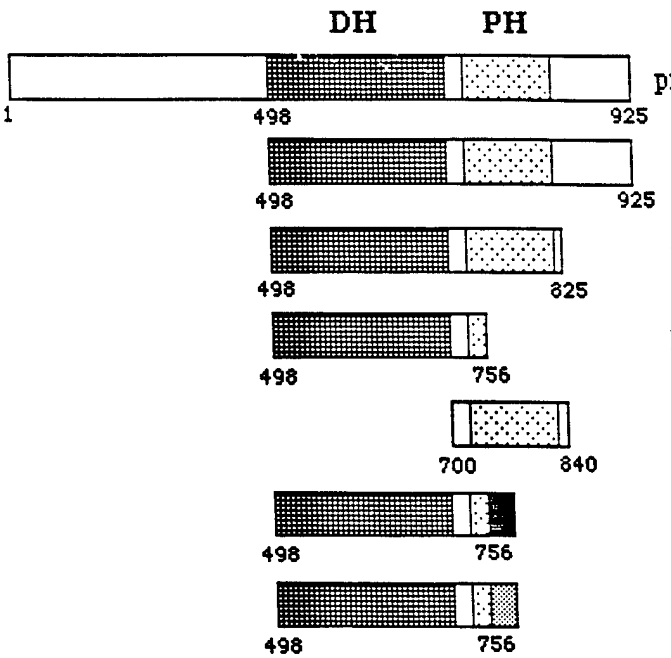
Figure 1. Schematic representations of oncogenic Dbl and different mutants of Dbl used in this study. The abilities of these constructs to serve as GEFs for Rho and Cdc42Hs and to transform NIH 3T3 cells are summarized. pdDH represents the Dbl homology domain and pdPH is the Pleckstrin homology domain. The abbreviation ffu represents focus forming units. 100% is 3 x $10^5$ foci/pmol DNA. pDHF is a construct in which the PH domain sequences downstream from residue 750 are replaced with the carboxyl terminal 16 amino acids of H-Ras, which include both the palmitylation and farnesylation sites (black rectangle). pdHF* is a construct encoding the DH domain of Dbl and the carboxyl terminal 16 amino acids of H-Ras, except that the cysteine which is normally farnesylated has been changed to serine (cross-hatched rectangle). The GEF activity for these constructs has not been determined (N/A). The pdDH, pdDHF, and pdDHF* were subcloned from *dbl* by PCR and inserted into the mammalian expression vector pZipneo. The GEF activities were measured as described (5) by the *in vitro* nitrocellulose filter binding assay either using the anti-Dbl immunoprecipitates from the NIH 3T3 transfectants (proto-Dbl, Ddbl, and pMA4) or using the insect cell expressed peptides (DH).

Figure 2. Expression of the PH domain inhibits Dbl-induced transformation in NIH 3T3 cells. A, Detection of the Dbl oncoprotein (using an anti-Dbl antibody) in NIH 3T3 transfectants. Lane 1 represents cells expressing the Dbl oncoprotein. Lane 2 represents cells co-expressing Dbl and the Dbl PH domain (dPH). Lane 3 represents cells co-expressing Dbl and the Vav PH domain. Lane 4 is a control, i.e. cells transfected with the plasmid (pFlag) used to express the PH domains. B, Detection of the PH domains of Dbl and Vav (using M5 anti-Flag antibody) in NIH 3T3 cells. Lane 1 represents cells co-expressing Dbl and the Dbl PH domain (dPH). Lane 2 represents cells co-expressing Dbl and the Vav PH domain. Lane 3 is a control (cells transfected with the pFlag plasmid). C, Effects of the PH domains of Dbl and Vav on Dbl-induced foci-formation. pFlag/PHdbl and pKH3/PHdbl represent the mammalian expression vectors encoding the Dbl PH

domain and pFlag/PHvav and pKH3/PHvav are the expression vectors encoding the Vav PH domain. The results shown represent the average of three independent experiments. D, Effects of PH domains on the anchorage-independent growth of Dbl transfectants.

Figure 3. The PH domain is not directly involved in the regulation of the GEF activity of the Dbl oncoprotein. A, Comparison of the abilities of oncogenic Dbl (■) and the DH domain of Dbl (▲) to stimulate [$^3$H]GDP dissociation of RhoA. The dissociation of [$^3$H]GDP from RhoA, alone, is depicted by (❑). B, Effect of the PH domain on the kinetics of Dbl-stimulated [$^3$H]GDP dissociation from RhoA. (■) represents Dbl-stimulated GDP dissociation in the absence of the PH domain and (◆) represents Dbl-stimulated GDP dissociation in the presence of the PH domain. (❑) and (◇) represent the corresponding controls for RhoA in the absence of Dbl.

Figure 4. The PH domain mediates the cytoskeletal association of the Dbl oncoprotein. A. The membrane attachment signal from H-Ras targets the DH domain to the Triton X-100 soluble fraction from cell membranes. pZIPneo/DHF (see Figure 1) represents the construct encoding the DH domain of Dbl and the carboxyl terminal 16 amino acids of H-Ras including the palmitylation and farnesylation sites. pZIPneo/DHF* represents the construct encoding the DH domain of Dbl and the carboxyl terminal 16 amino acids of H-Ras (except that the cysteine which serves as the farnesylation site has been changed to serine). S represents the soluble fraction, T is the Triton X-100 soluble fraction from membranes, and I is the Triton X-100 insoluble fraction. B, Oncogenic Dbl is associated with the Triton X-100 insoluble fraction of cells. C, Fractionations of the pMA4 and DH domain transfectants. D, Fractionation of cells expressing the Dbl PH domain. The data shown in A-C were obtained by immunoprecipitating the Dbl proteins with the anti-Dbl antibody from cells that were labeled with [$^{35}$S]methionine and [$^{35}$S]cysteine. The data shown in D represents immunoblots using the anti-Flag M5 antibody.




| | GEF activity | ffu/pmol DNA % of control |
|---|---|---|
| proto-dbl | + | 1.7 |
| dbl | + | 100 |
| pMA4 | + | 98 |
| pdDH | + | <0.001 |
| pdPH | - | <0.001 |
| pdDHF | N/A | <0.001 |
| pdDHF* | N/A | <0.001 |

Figure 1

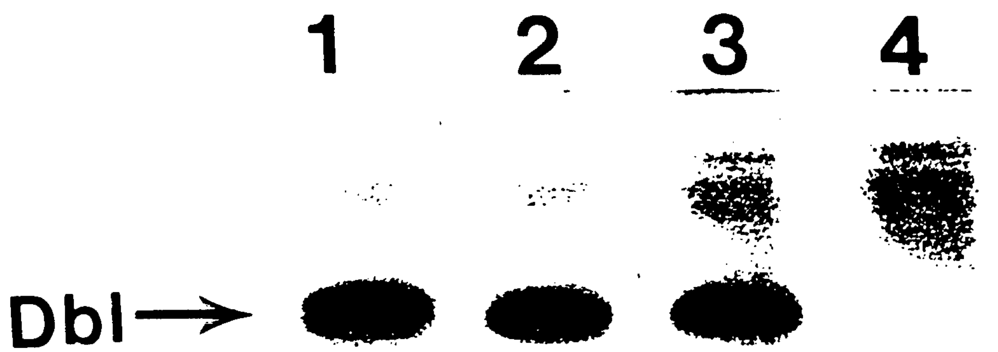



Figure 2A

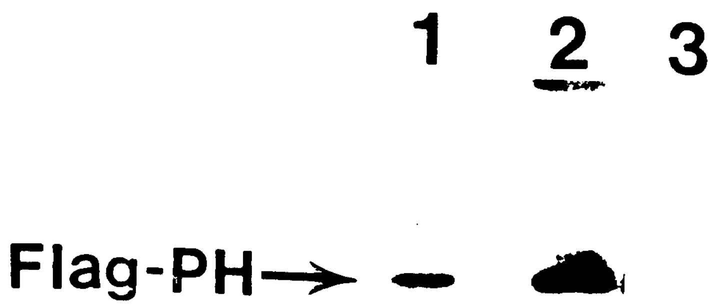



Figure 2B

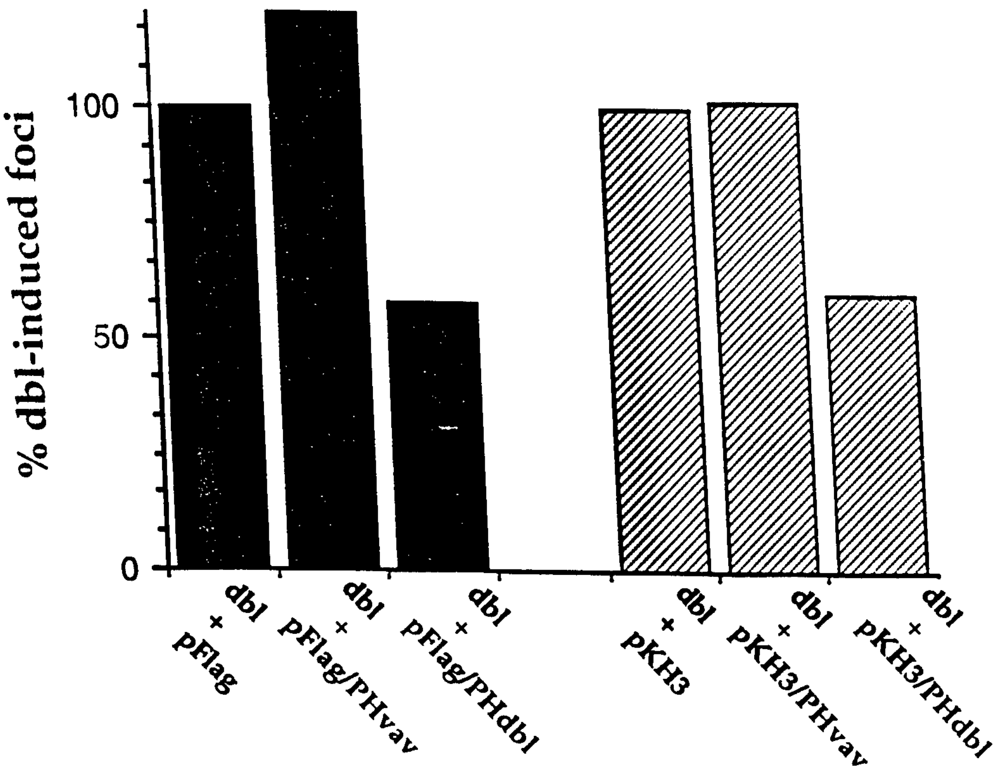

% dbl-induced foci
100
50
0
dbl + pFlag
dbl + pFlag/PHvav
dbl + pFlag/PHdbl
dbl + pKH3
dbl + pKH3/PHvav
dbl + pKH3/PHdbl

Figure 2C

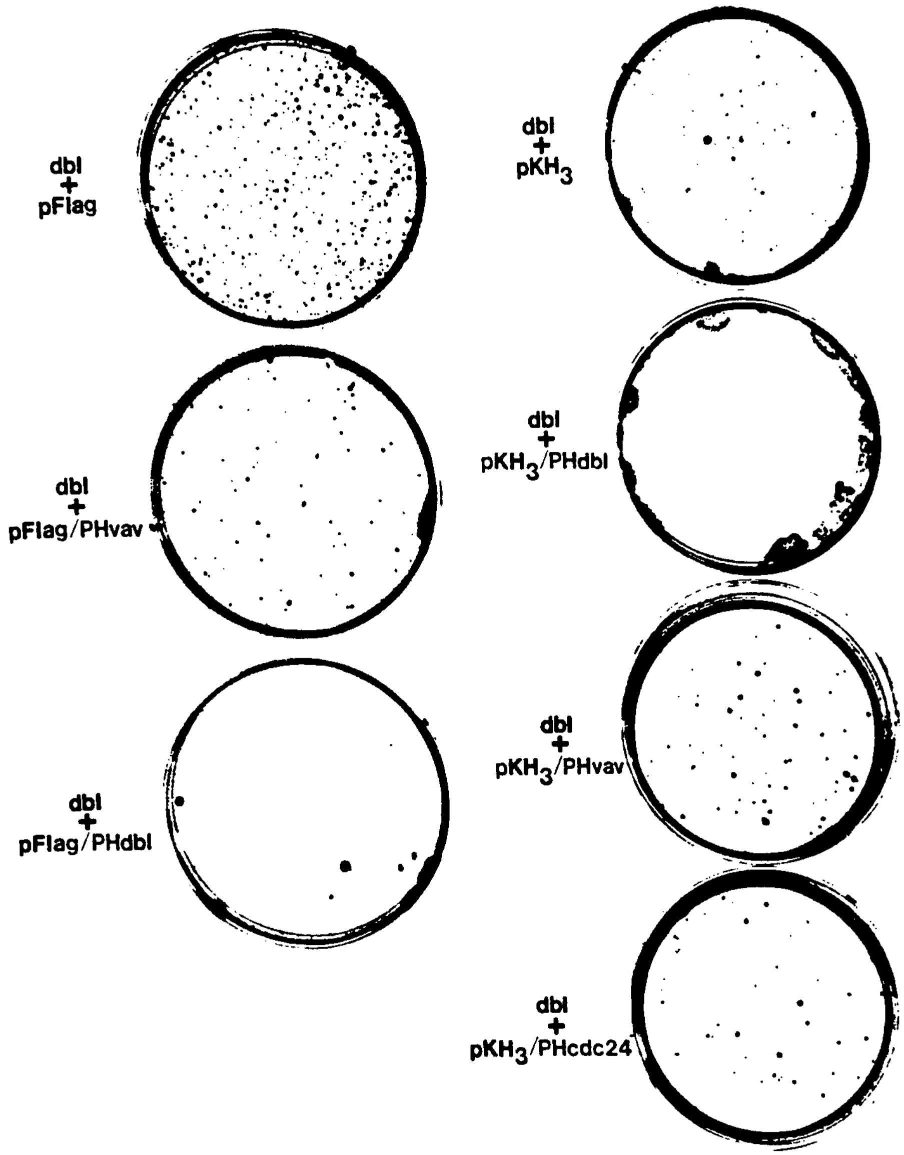

dbl
+
pFlag
dbl
+
pKH3
dbl
+
pKH3/PHdbl
dbl
+
pFlag/PHvav
dbl
+
pKH3/PHvav
dbl
+
pFlag/PHdbl
dbl
+
pKH3/PHcdc24

Figure 2D

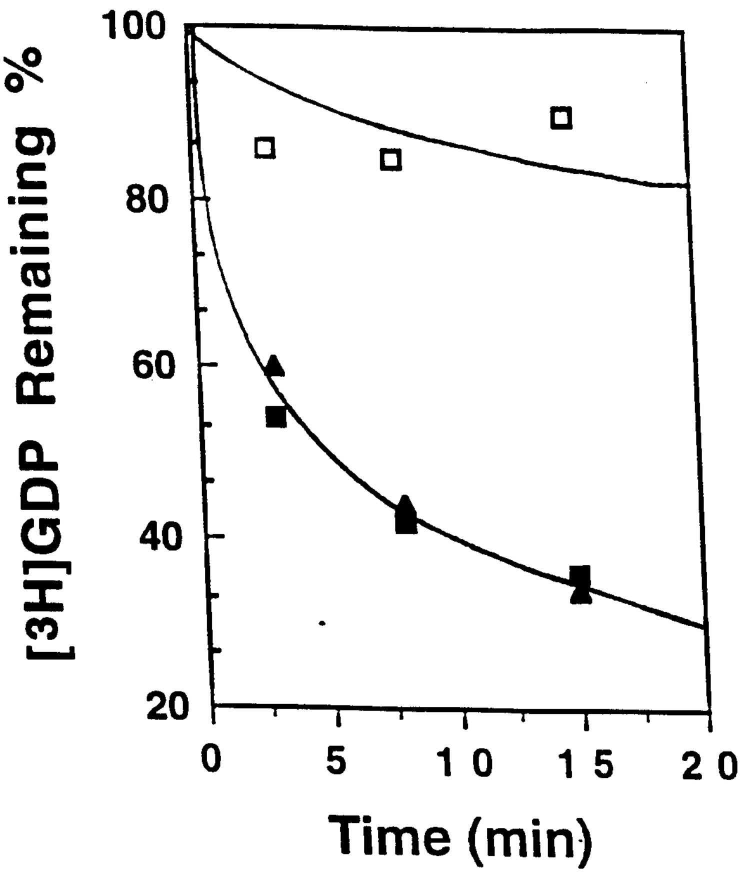



Figure 3A

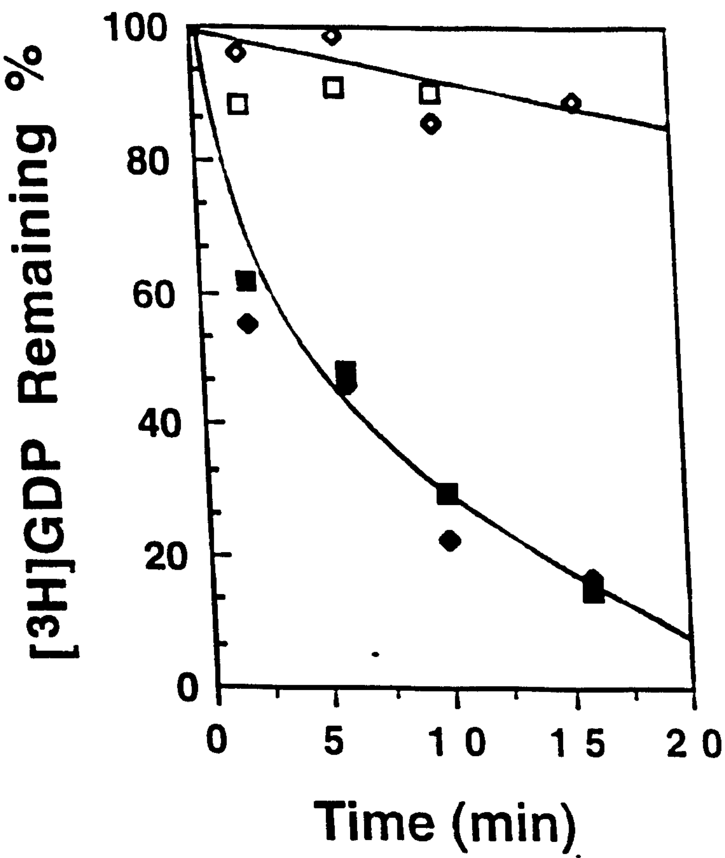



Figure 3B

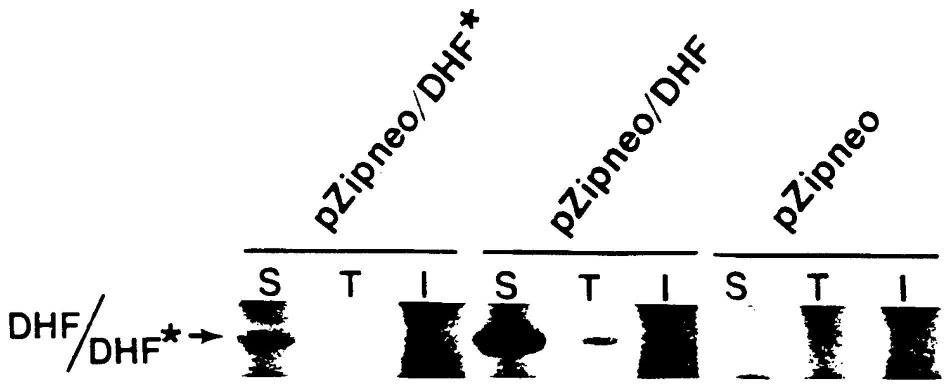



**Figure 4A**

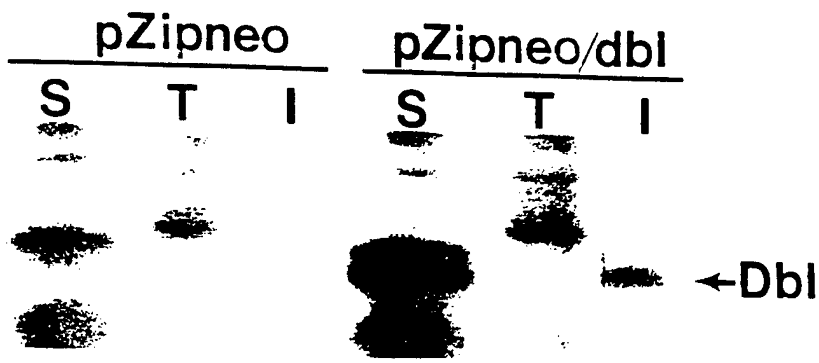



Figure 4B


Figure 4C


Figure 4D

# Phosphatidylinositol 4,5 bisphosphate ($PIP_2$) provides an alternative to guanine nucleotide exchange factors by stimulating the dissociation of GDP from Cdc42Hs

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## ABSTRACT

Members of the Rho-subfamily of Ras-related GTP-binding proteins play important roles in the organization of the actin cytoskeleton and in the regulation of cell growth. We have previously shown that the *dbl* oncogene product, which represents a prototype for a family of growth regulatory proteins, activates Rho-subfamily GTP-binding proteins by catalyzing the dissociation of GDP from their nucleotide binding site. In the present study, we demonstrate that the acidic phospholipid, phosphatidylinositol 4,5 bisphosphate ($PIP_2$), provides an alternative mechanism for the activation of Cdc42Hs. Among a panel of 10 lipids tested, only $PIP_2$ was able to stimulate GDP release from Cdc42Hs in a dose-dependent manner, with a half maximum effect at ~50 μM. Unlike the Dbl oncoprotein, which requires the presence of (free) guanine nucleotide in the medium in order to replace the GDP bound to Cdc42Hs, $PIP_2$ stimulates GDP release from Cdc42Hs in the absence of free guanine nucleotide. $PIP_2$, when incorporated into phosphatidylcholine carrier vesicles, binds tightly to the guanine nucleotide-depleted form of Cdc42Hs, weakly to the GDP-bound form of the GTP-binding protein, but does not bind to GTP-bound Cdc42Hs, similar to what was observed for the Dbl oncoprotein. However, mutational analysis of Cdc42Hs indicates that the site of specific interaction between $PIP_2$ and Cdc42Hs is distinct from the Dbl-binding site and is located at the positively charged carboxyl-terminal end of the GTP-binding protein. The GDP-releasing activity of $PIP_2$ is highly effective toward Cdc42Hs and Rho [and is similar to the reported effects of $PIP_2$ on Arf (Terui, T. et al., J. Biol. Chem. (1995) 270, 17656-17659)], is less effective with Rac, and is not observed with Ras, Rap1a, or Ran. The ability of $PIP_2$ to activate Cdc42Hs (or Rho) and Arf provides a possible point of convergence for the biological pathways regulated by these different GTP-binding proteins and may be related to the synergism observed between Arf and Rho-subtype proteins in the stimulation of phospholipase D activity (Singer, W., et al., J. Biol. Chem. (1995) 270, 14944-14950).

## INTRODUCTION

The Rho subfamily of Ras-related GTP-binding proteins, which includes RhoA, Rac1, and Cdc42Hs, has been shown to regulate a diversity of cellular functions ranging from actin-mediated cytoskeletal rearrangements (Hall, 1994) to the stimulation of nuclear MAP kinases (Coso et al., 1995; Minden et al., 1995; Bagrodia et al., 1995), transcription (Hill et al., 1995), and DNA synthesis (Olson et al., 1995). It is now well established that Rac1 is essential for growth factor-stimulated membrane ruffling and lamellipodia formation (Ridley et al., 1992) and acts downstream from Ras in the stimulation of cell growth (Qui et al., 1995), while RhoA controls the formation of stress fibers and focal adhesion complexes (Ridley and Hall, 1992). Cdc42 has been shown to be essential for bud-site assembly in *Saccharomyces cerevisiae* (Johnson and Pringle, 1990), for uni-directional and bi-directional cell growth in *Schizosaccharomyces pombe* (Miller and Johnson, 1994), and for filopodia formation in mammalian cells (Nobes and Hall, 1995; Kozma et al., 1995). The macromolecular targets for the Rho-subfamily GTP-binding proteins are just now beginning to be identified. Both GTP-bound Rac1 and Cdc42Hs bind to the 85 kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase (PI 3-kinase) (Zheng et al., 1994a; Tolias et al., 1995) and to members of the p21-activated serine/threonine kinase (PAK) family (Manser et al., 1994; Martin et al., 1995; Bagrodia et al., 1995). The GTP-bound form of RhoA also has been reported to stimulate PI 3-kinase activity in platelets (Zhang et al, 1993) as well as PI 4-phosphate 5-kinase activity (Chong et al., 1994). In addition, it recently has been shown that RhoA and Cdc42Hs can act synergistically with Arf to stimulate phospholipase D activity (Singer et al., 1995).

Understanding how these different target activities are stimulated and then integrated to yield cytoskeletal changes and nuclear activities represents a formidable challenge. One approach is to determine how Rho-subfamily GTP-binding proteins are activated, since this should represent the first key step in stimulating their target activities. One mode of activation of Rho-subtype GTP-binding proteins occurs through the stimulation of GDP dissociation (and consequently GTP/GDP

exchange) by the family of Dbl-related proteins. The prototype for this family of guanine nucleotide exchange factors (GEFs) is the Dbl oncoprotein, which was shown to act as a GEF for Cdc42Hs and RhoA (Hart et al., 1991; Hart et al.; 1994). At present, 15 members of the Dbl-related family have been identified (Cerione and Zheng, 1996), with each member containing the characteristic Dbl-homology (DH) domain in tandem with a Pleckstrin-homology (PH) domain. Many of these proteins have been shown to have GEF activity including Cdc24 (Zheng et al., 1994b), Ost (Horii et al., 1994), Tiam-1 (Michiels et al., 1995), and Lbc (Zheng et al., 1995). Similar to the case for the interactions between heterotrimeric GTP-binding proteins (G proteins) and agonist-stimulated heptahelical receptors (Gilman, 1987), the Dbl-related GEFs bind preferentially to, and stabilize, the guanine nucleotide-depleted states of Rho-like GTP-binding proteins.

It is interesting that many of these Dbl-related proteins show a selective tissue distribution, whereas many of the Rho-subtype proteins (e.g. Cdc42Hs, RhoA, Rac1) appear to be ubiquitous. This suggests that additional but as yet undiscovered Dbl-related proteins exist and/or that other mechanisms may be utilized to stimulate the activation of Rho-subtype GTP-binding proteins. In the present study, we describe one such potential alternative mechanism where the lipid phosphatidylinositol 4,5 bisphosphate ($PIP_2$) is able to strongly stimulate GDP dissociation from Cdc42Hs and RhoA.

## EXPERIMENTAL PROCEDURES

*Materials* - The small GTP-binding proteins Cdc42Hs, RhoA, Rac1, and H-Ras were expressed and purified as glutathione-S-transferase (GST) fusion proteins from *E. coli* as described previously (Hart et al., 1994). The Rap1a protein was a kind gift from Dr. P. Polakis (Onyx Pharmaceutical, Emoryville, CA). The cDNA encoding Ran was the generous gift of Dr. M. Rush (NYU Medical Center, New York, NY). In order to express Ran in *E coli* as a glutathione-S-transferase (GST) fusion protein, restriction sites for NcoI and HindIII were introduced immediately adjacent to the initiation and termination codons, respectively, using the polymerase chain reaction (PCR). The PCR product (730 bp) was then ligated with NcoI/HindIII-digested pGEX-KG (Pharmacia) and the resultant GST-Ran fusion protein was purified from transformed *E. coli* (JM101). Automated DNA sequencing revealed no mutations in the PCR product. The anti-Cdc42Hs polyclonal antibodies were raised against the unique carboxyl terminal sequences of Cdc42Hs as described (Shinjo et al., 1991). GST-Dbl protein was expressed in a baculovirus/Sf9 insect cell system (Hart et al., 1994), and the Cdc42Hs-GAP was expressed and purified from *E. coli* (Barfod et al., 1993). The ΔC7 Cdc42Hs truncation mutant was generated by PCR using the Pfu DNA polymerase (Stratagene), and the resulting sequences were verified through fluorescence automated sequencing. Lipids were purchased either from Sigma or from Avanti Polar Lipids. All lipids were dissolved in chloroform, dried under nitrogen, and resuspended by sonication in 50 mM Tris-HCl (pH 8.0) immediately prior to use. The pH was adjusted as necessary with NaOH. Radioisotope-labeled guanine nucleotides were obtained from DuPont, NEN.

*GDP dissociation and GTP/GDP Exchange Assays* - GDP dissociation and GTP/GDP exchange assays were carried out as previously described (Hart et al., 1991). 2 μg of Cdc42Hs loaded with [$^3$H]GDP was incubated with buffer mixtures containing 100 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 5 mM $MgCl_2$ (buffer A) with various lipids or Dbl proteins for the indicated times at room temperature. Assays monitoring the dissociation of GDP were stopped by dilution

(20 μl aliquots) into 10 ml ice-cold buffer A, and the protein-bound nucleotide was trapped by filtration on nitrocellulose filters. For GTP/GDP exchange assays, 1 mM GTP was also included in the reaction buffer.

*GTPγS Binding Assays* - Assays monitoring the dissociation of GTPγS from Cdc42Hs were performed as described above for GDP, except that [$^{35}$S]GTPγS was used and the concentration of Cdc42Hs-GTPγS was ~0.4 μM. GTPγS binding was determined as in Hart et al. (1991). 2 μg of GDP-bound Cdc42Hs were incubated in buffers containing 100 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 10 μM [$^{35}$S]GTPγS (~5,000 cpm/pmol), and 100 μM $PIP_2$, 0.5 μM GST-Dbl, or 100 μM phosphatidylcholine (PC) at 24°C, and binding was determined at various time points.

*Liposome-protein Complex Formation Assays* - Direct binding of liposome vesicles containing $PIP_2$ to Cdc42Hs was carried out by adapting the centrifugation protocol by Harlan et al. (1994). Briefly, Cdc42Hs was first loaded with GDP or GTPγS, or depleted with nucleotide (Hart et al., 1994). 1 μg of Cdc42Hs was then added to lipid vesicles (100 μl total volume) with 500 μM carrier PC alone or 100 μM $PIP_2$ incorporated into the PC carrier vesicles through co-sonication. The mixture was incubated for 5 minutes before centrifugation in an ultraspeed Beckman airfuge (100,000g) for 30 minutes. The vesicles pelleted with this treatment were subjected to SDS-PAGE and anti-Cdc42Hs Western blot analysis, and the blot was visualized by the ECL method (DuPont, NEN).

## RESULTS AND DISCUSSION

The mode of regulation of Rho-family GTP-binding proteins has been an area of intense research investigation (Boguski and McCormick, 1993), due to the involvement of these GTP-binding proteins in the stimulation of cytoskeletal changes and transcriptional activities as well as in the regulation of cell growth. In addition to protein factors that directly stimulate the guanine nucleotide exchange activities of Rho-subtype GTP-binding proteins, for which the Dbl oncoprotein is a prototype (Hart et al., 1994), various phospholipids have been implicated in the regulation of the Rac GTP-binding proteins through their effects on the GDI molecule (Chuang et al., 1993). In addition, the GTP-binding protein Arf, which undergoes GDP dissociation in response to $PIP_2$, appears to act synergistically with Rho-subtype proteins to stimulate (in a $PIP_2$-sensitive manner) phospholipase D activity (Malcolm et al., 1994; Singer et al., 1995; Moss and Vaughan, 1995). The latter finding is particularly interesting since we recently have found that Cdc42Hs is predominantly localized to Golgi membranes in mammalian cells and that its localization is influenced by different Arf mutants in a manner suggesting some type of interplay between Arf and Cdc42Hs (Erickson et al.)[2]. Given these findings, we examined whether various phospholipids had any effect on the GTP-binding/GTPase cycle of Cdc42Hs.

Figure 1A shows the results obtained when examining the effects of a panel of phospholipids on the rate of [$^3$H]GDP dissociation from Cdc42Hs. Only $PIP_2$ showed a significant stimulation of GDP dissociation from Cdc42Hs. Essentially no effect was observed when phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylserine (PS), or phosphatidylinositol (PI) were incubated with [$^3$H]GDP-bound Cdc42Hs. However, PIP, at concentrations > 500 μM, caused a slight increase in the rate of [$^3$H]GDP dissociation (data not shown).

Figure 1B shows that the $PIP_2$-stimulated dissociation of GDP from Cdc42Hs is dose-dependent with a half maximal effect occurring at ~ 50 μM $PIP_2$. At $PIP_2$ levels ≥ 200 μM, the rate of GDP release from Cdc42Hs was increased ~10 fold.

The stimulation of GDP dissociation by $PIP_2$ was similar to that elicited by the oncogenic Dbl protein (Hart et al., 1994). Figure 2A shows time courses for the dissociation of [$^3$H]GDP from Cdc42Hs in the absence of activators or in the presence of the Dbl oncoprotein (~0.5 μM) or $PIP_2$ (100 μM). In these experiments, the dissociation of GDP was assayed in the presence of 1 mM GTP in the medium. Under these conditions, the half time for [$^3$H]GDP dissociation from Cdc42Hs (in the presence of phosphatidylcholine as a control) was ~25 minutes, whereas Dbl-catalyzed GDP dissociation occurred with a half time of ~1.5 minutes and $PIP_2$-stimulated GDP dissociation occurred with a half time of ~2.5 minutes.

It is interesting that when GDP dissociation was assayed in the absence of medium GTP, dramatic differences were observed between Dbl and $PIP_2$ (Figure 2B). Specifically, $PIP_2$ was still capable of providing a strong stimulation of the initial rate of [$^3$H]GDP dissociation, whereas oncogenic Dbl failed to induce any detectable stimulatory effect.

These findings can be considered within the context of the generally accepted model for the interactions of guanine nucleotides and guanine nucleotide exchange factors (GEFs) with GTP-binding proteins. It typically has been assumed that GEFs act as antagonists of guanine nucleotide binding and that conversely, nucleotide binding weakens the interactions of GEFs with GTP-binding proteins. Thus, although Dbl weakens the affinity of GDP for Cdc42Hs, the amount of GDP present (i.e. initially bound to Cdc42Hs) is sufficient to maintain occupancy of the nucleotide site, even in the presence of this GEF. However, when a high excess of GTP is included in the medium, it effectively competes with GDP for the nucleotide site, thereby resulting in Dbl-catalyzed GTP-GDP exchange. This differs from the case for $PIP_2$, where this putative GEF is apparently more effective than Dbl in weakening the affinity of Cdc42Hs for guanine nucleotides. $PIP_2$ strongly stabilizes the guanine nucleotide-depleted state of Cdc42Hs and thus it is difficult to observe a $PIP_2$-stimulated exchange of GDP for [$^{35}$S]GTPγS. In fact, under conditions where Dbl is able to elicit a strong stimulation of [$^{35}$S]GTPγS binding (when [GTPγS] = 0.5-1 μM), the addition of $PIP_2$ shows no detectable stimulation of GTPγS binding (Figure 2C). In addition, while $PIP_2$ is more effective than Dbl in weakening the binding of guanine nucleotides to

Cdc42Hs, it appears that guanine nucleotides also weaken the binding of $PIP_2$ to a greater extent than the binding of Dbl. Thus, while Dbl shows a weak but measurable stimulation of [$^{35}$S]GTP$\gamma$S dissociation from Cdc42Hs, the addition of $PIP_2$ (at 50-100 $\mu$M) has no effect (data not shown).

Taken together, the results presented in Figure 2 would suggest that $PIP_2$ is capable of a direct interaction with Cdc42Hs and that this interaction is significantly influenced by the guanine nucleotide bound to the GTP-binding protein. Figure 3 shows the results of such a direct binding experiment, using phosphatidylcholine (PC) vesicles containing $PIP_2$. In this experiment, PC vesicles alone and PC vesicles containing $PIP_2$ were first incubated with Cdc42Hs, either in the guanine nucleotide-depleted state, or in the GDP-bound state or the GTP$\gamma$S-bound state, for 10 minutes at room temperature before pelleting the lipid vesicles by ultracentrifugation. Western blotting the pelleted vesicles with a specific anti-Cdc42Hs antibody revealed that Cdc42Hs, depleted of bound guanine nucleotide, was capable of tightly associating with the PC/$PIP_2$ vesicles, while Cdc42Hs containing bound GDP was capable of a weaker association with these vesicles. The GTP$\gamma$S-bound Cdc42Hs showed no ability to associate with the PC/$PIP_2$ vesicles (i.e. relative to the background association observed with control PC vesicles). Thus, $PIP_2$ shows the same pattern of association with different guanine nucleotide-bound forms of Cdc42Hs as originally observed for the Dbl oncoprotein (Hart et al., 1994).

It had been earlier reported that $PIP_2$ was able to stimulate the dissociation of [$^{3}$H]GDP from the Arf GTP-binding proteins (Terui et al., 1994). Given our findings with Cdc42Hs, we were interested in determining whether $PIP_2$ might serve as a potential activator of other GTP-binding proteins, and in particular, other members of the Rho-subfamily. As shown in Figure 4, we found that $PIP_2$ was most effective on Cdc42Hs and RhoA, stimulating the dissociation of greater than 80% of the total bound [$^{3}$H]GDP within 5 minutes at room temperature. $PIP_2$ showed some ability to stimulate GDP dissociation from Rac1 (~50% of the total bound [$^{3}$H]GDP was dissociated after 5 minutes) but no ability to stimulate GDP dissociation from the Ras, Rap1a, or Ran GTP-binding proteins (Figure 4), even when using $PIP_2$ levels as high as 0.5 mM.

The ability of $PIP_2$ to regulate nucleotide binding to Rho-subfamily proteins, as well as Arf, and to directly associate with Cdc42Hs (see Figure 3), suggested that the Rho-subfamily GTP-binding proteins must contain a specific $PIP_2$-binding site. Sequence comparisons between Rho-subfamily GTP-binding proteins and two $PIP_2$-binding proteins, Gelsolin and Villin, indicated that the carboxyl terminal domains of Cdc42Hs, RhoA, and the Rac proteins, which contain a number of basic amino acids, shared homology with amino acid residues 140-147 of Villin and 150-169 of Gelsolin. These regions of Villin and Gelsolin have been implicated in the binding of these proteins to $PIP_2$ (Jamney et al., 1992). Thus, we constructed a deletion mutant of Cdc42Hs that lacked the carboxyl terminal 7 amino acids (including two arginines that were suspected to be involved in $PIP_2$ binding). This truncated Cdc42Hs molecule behaves like wild type Cdc42Hs with regard to its intrinsic GTP-binding and GTPase activities and its ability to functionally couple to the Cdc42Hs-GAP (data not shown). While it undergoes a slower rate of [$^3$H]GDP dissociation, compared to wild type Cdc42Hs, it is still capable of interacting with Dbl and undergoing Dbl-catalyzed GDP dissociation (Figure 5). However, the carboxyl terminal truncated Cdc42Hs shows a markedly reduced response to $PIP_2$. These results then strongly argue that while Dbl and $PIP_2$ elicit similar effects (i.e. stimulation of GDP dissociation), they mediate these common effects from distinct binding domains on the GTP-binding protein.

In addition to serving as a precursor for the second messengers $IP_3$ and diacylglycerol (Berridge, 1993) and for the putative messenger $PIP_3$ (Stephens et al., 1993), $PIP_2$ has been implicated as a regulator of the actin cytoskeleton, based on its ability to influence actin severing, capping, and bundling proteins *in vitro* (Janmey, 1994). Thus, it is interesting that in the present studies, we find that $PIP_2$ binds directly to and influences the nucleotide state of GTP-binding proteins that have been implicated in cytoskeletal regulation, namely Cdc42Hs and RhoA. However, these findings raise a number of important issues. One has to do with the mechanism by which $PIP_2$ stimulates GDP dissociation and how this compares with the mechanism by which Dbl stimulates GDP dissociation and guanine nucleotide exchange. Certainly the rate-limiting step in the activation of GTP-binding proteins, which occurs as an outcome of the exchange of GTP for

bound GDP, is the dissociation of the tightly bound GDP molecule. Both Dbl and $PIP_2$ strongly catalyze this dissociation event and stabilize the nucleotide-depleted state of the GTP-binding protein. Our data, in fact, would suggest that $PIP_2$ does this even more effectively than Dbl, such that $PIP_2$ can stimulate GDP dissociation from the nucleotide binding site of Cdc42Hs in the absence of added GTP, whereas Dbl cannot. It is possible that the differences exhibited by $PIP_2$ and Dbl reflect differences in the sites on Cdc42Hs (or related proteins) that bind these agents. At the present time, we know very little about the specific sites on Cdc42Hs that are responsible for binding Dbl, although mutations in Cdc42Hs that correspond to mutations in Ras that uncouple its binding to the GEF, Sos (Mosteller et al., 1994), do not uncouple Cdc42Hs from Dbl (Y. Zheng, unpublished results). We would speculate at this point that a key conformational change that is necessary to loosen the binding of GDP to Cdc42Hs is induced by both Dbl and $PIP_2$ from distinct (binding) sites on the Cdc42Hs molecule. Future studies will be aimed obtaining additional information regarding the conformational change in Cdc42Hs and related Rho-subfamily proteins that is necessary for this rate-limiting step for activation.

A second key issue raised by these studies concerns whether in fact $PIP_2$ acts as a physiological regulator of Cdc42Hs and related proteins, and if so, how? It is tempting to speculate that the actin regulatory activities of $PIP_2$ are related to the actions of Cdc42Hs and RhoA in mediating cytoskeletal changes such as filopodia formation and/or actin stress fiber formation. This is a particularly interesting possibility given the suggestions that a cascade of Rho-subfamily GTPases (i.e. Cdc42Hs-Rac1-RhoA) is operating in the regulation of cytoskeletal changes in certain cells (Nobes and Hall, 19995) and that a putative target for Cdc42Hs, the PI 3-kinase (Zheng et al., 1994a), which generates phosphatidylinositol compounds phosphorylated at the 3 position, may be upstream from Rac1 (Hawkins et al., 1995; Nobes et al., 1995). Thus, in addition to Dbl-related proteins, it is possible that PI metabolites might serve as direct regulators of a signaling cascade that leads to changes in the actin cytoskeleton. In addition, studies with purified phospholipase D indicate that the maximum activation of at least one isoform of the enzyme requires $PIP_2$ and the synergistic actions of the Arf GTP-binding protein [which also binds

and is regulated by $PIP_2$ (Terui et al., 1995)] and either RhoA or Cdc42Hs (Singer et al., 1995). In the future, we intend to examine these possibilities further as well as determine just how $PIP_2$ levels in cells might be coordinated with the activation-deactivation cycle of Cdc42Hs and/or RhoA. For example, it is possible that signaling pathways that lead to an increase in $PIP_2$ levels will also promote the generation of guanine nucleotide-depleted Cdc42Hs and/or RhoA. Situations which then lead to a decrease in $PIP_2$ levels (e.g. stimulation of the hydrolysis of $PIP_2$ by phospholipase C enzymes) would then enable cellular GTP to bind to these GTP-binding proteins, thereby stimulating their activation. However, it also is possible that at the cellular levels of GTP (> 10 μM), the exchange of GDP for GTP can occur even in the presence of high concentrations of $PIP_2$. Thus far it has been difficult to test these levels of GTP in conventional GTP-binding assays; however, we hope in the future to be able to use fluorescence spectroscopic approaches to determine if in fact such a $PIP_2$-stimulated nucleotide exchange reaction is feasible.

## Footnotes

[1]The abbreviations used are: GAP, GTPase-activating protein; GDI, GDP-dissociation inhibitor; GEF, guanine nucleotide exchange factor; MAP, mitogen-activated protein; $PIP_2$, phosphatidylinositol 4,5 bisphosphate; PI 3-kinase, phosphatidylinositol 3-kinase; GST, glutathione S-transferase; GTPγS, guanosine 5'-3-O-(thio)triphosphate.

[2]Erickson, J.W., Zhang, C.-J., Kahn, R.A., Evans, T., and Cerione, R.A. "Mammalian Cdc42 is a brefeldin A sensitive component of the Golgi apparatus", submitted.

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## FIGURE LEGENDS

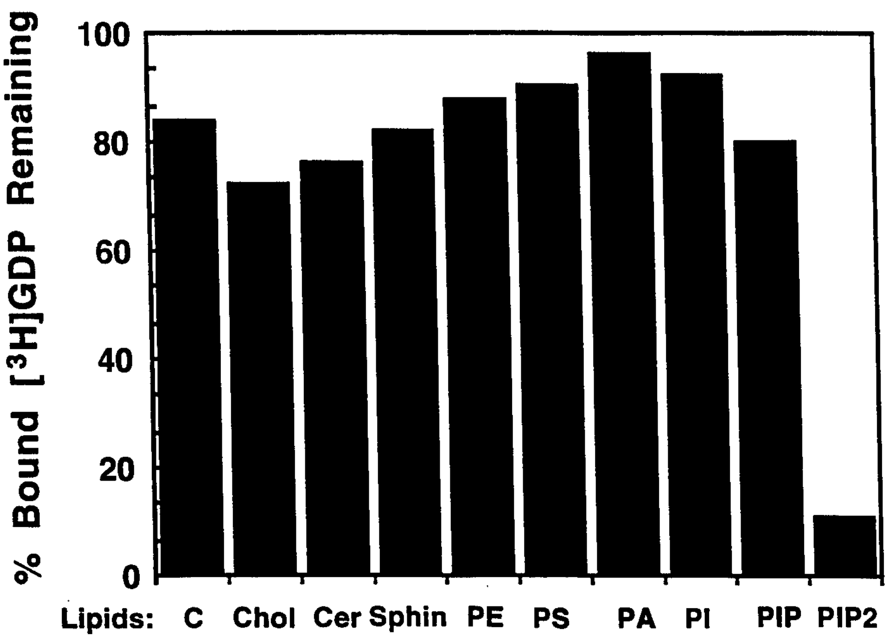
Figure 1. Effects of lipids on guanine nucleotide dissociation from Cdc42Hs. 1A, Purified lipids at a concentration of 200 μM in buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, and 5 mM $MgCl_2$, were added to the incubation mixture with 2 μg *E. coli*-expressed [$^3$H]GDP-Cdc42Hs at a final concentration of 100 μM and the reactions were terminated after 8 minutes at room temperature. 1B, Dose dependent [$^3$H]GDP dissociation from Cdc42Hs stimulated by $PIP_2$. GDP dissociation reactions were terminated 10 minutes after mixing $PIP_2$ and [$^3$H]GDP-bound Cdc42Hs.

Figure 2. Comparisons of the guanine nucleotide exchange activities of Cdc42Hs stimulated by $PIP_2$ or the Dbl oncoprotein. 2A, Time courses of [$^3$H]GDP release from 2 μg Cdc42Hs stimulated by 100 μM $PIP_2$ (❑), 0.5 μM GST-Dbl (■), or 200 μM PC (◆) under the GTP/GDP exchange assay conditions (with 1 mM free GTP). 2B, Time courses of [$^3$H]GDP release from 2 μg Cdc42Hs stimulated by 100 μM $PIP_2$ (❑), 0.5 μM GST-Dbl (■), or 200 μM PC (◆) in the absence of free nucleotides. 2C, Effects of $PIP_2$ and Dbl on [$^{35}$S]GTPγS binding to Cdc42Hs. Cdc42Hs was pre-loaded with GDP and the time courses for the binding of [$^{35}$S]GTPγS to Cdc42Hs in the presence of 100 μM $PIP_2$ (❑), 0.5 μM GST-Dbl (■), or 200 μM PC (◆) were determined.

Figure 3. $PIP_2$ binds to the guanine nucleotide-depleted form of Cdc42Hs. 1 μg of nucleotide-depleted Cdc42Hs (EDTA lane), Cdc42Hs bound to GDP (GDP lane), or Cdc42Hs bound to GTPγS (GTPγS lane) was incubated with 100 μM $PIP_2$ incorporated in PC vesicles for 10 minutes before ultracentrifugation in an airfuge. The resulting pellets were subjected to an anti-Cdc42Hs Western blot. Lane (-), Cdc42Hs depleted of nucleotide incubated with PC vesicles alone; lane (+), 0.1 μg Cdc42Hs as a positive control.

Figure 4. $PIP_2$ stimulates GDP-dissociation from the Rho-family GTP-binding proteins, Cdc42Hs and RhoA. 1 μg of purified H-Ras, Rap1a, Ran, RhoA, Rac1, or Cdc42Hs were incubated with 100 μM $PIP_2$ under the GDP-dissociation assay conditions for 8 minutes.

Figure 5. The carboxyl-terminal domain of Cdc42Hs is necessary for its interaction with $PIP_2$. 1 μg of a carboxyl-terminal truncated (CΔ7) mutant of Cdc42Hs preloaded with $[^3H]$GDP was incubated with 100 μM $PIP_2$ (□), 0.5 μM GST-Dbl (■), or 1 μM GST (◆), and aliquots of the reaction mixtures were added to the termination buffers at the indicated time points.



% Bound [3H]GDP Remaining
100
80
60
40
20
0
Lipids: C Chol Cer Sphin PE PS PA PI PIP PIP2


Figure 1A

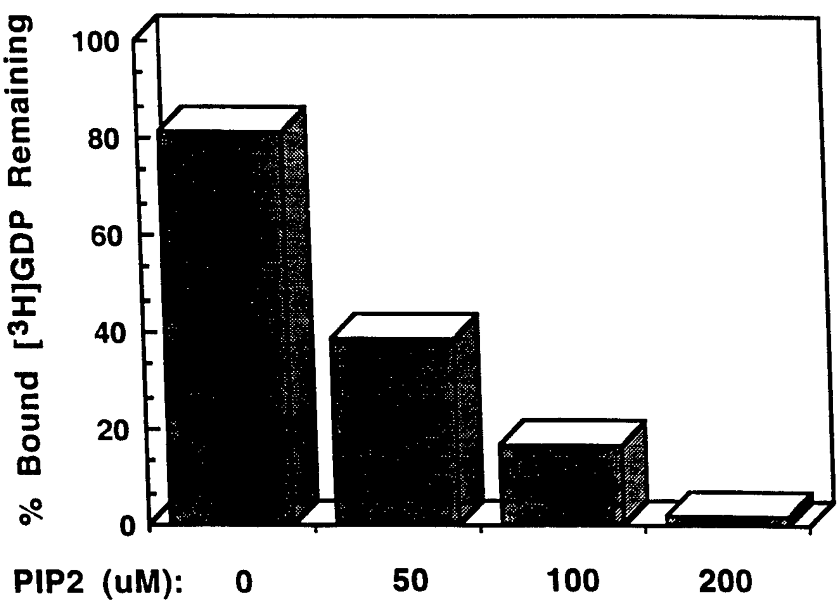



Figure 1B

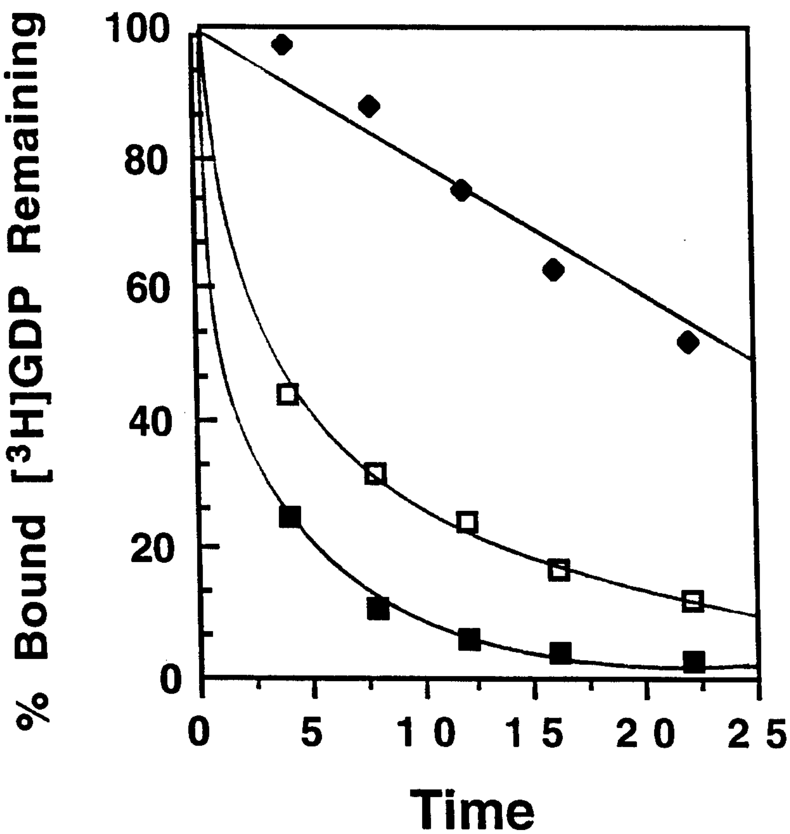



Figure 2A

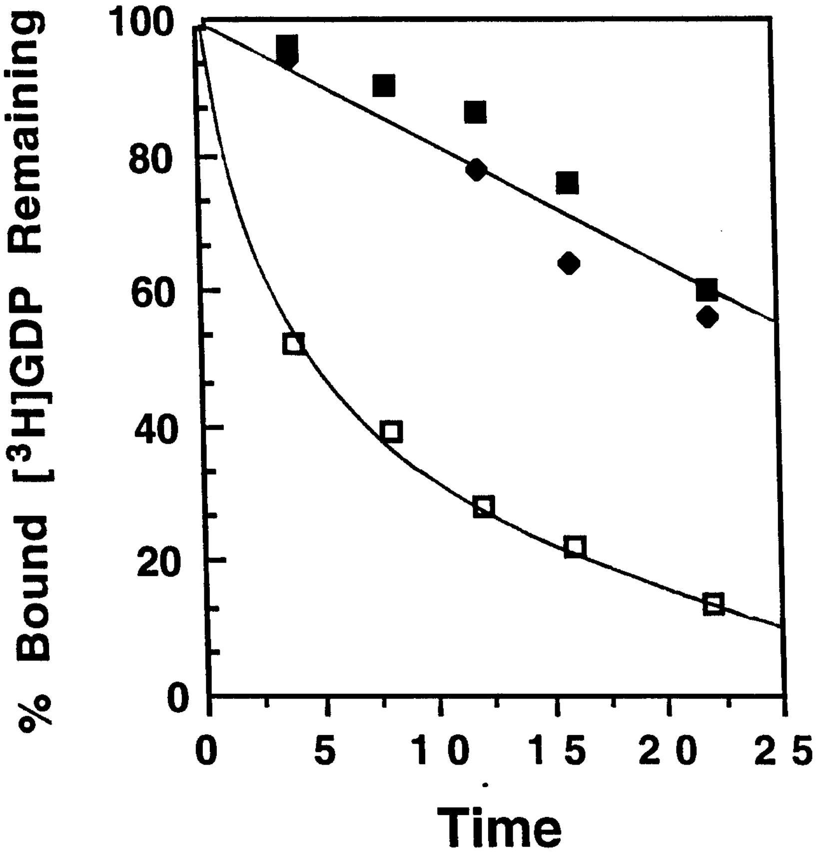



Figure 2B

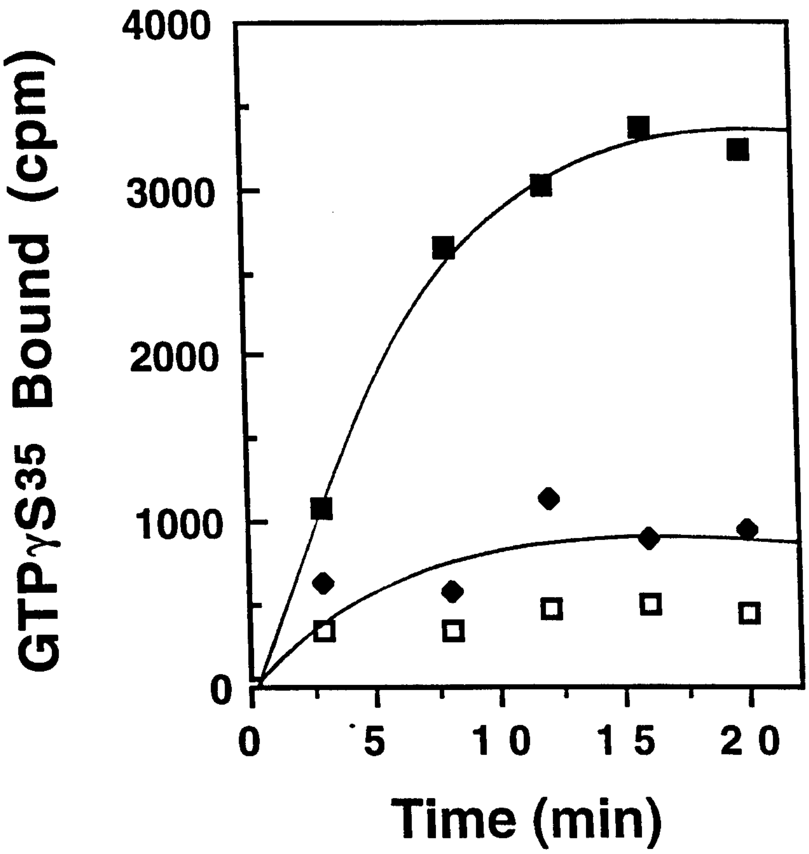



Figure 2C

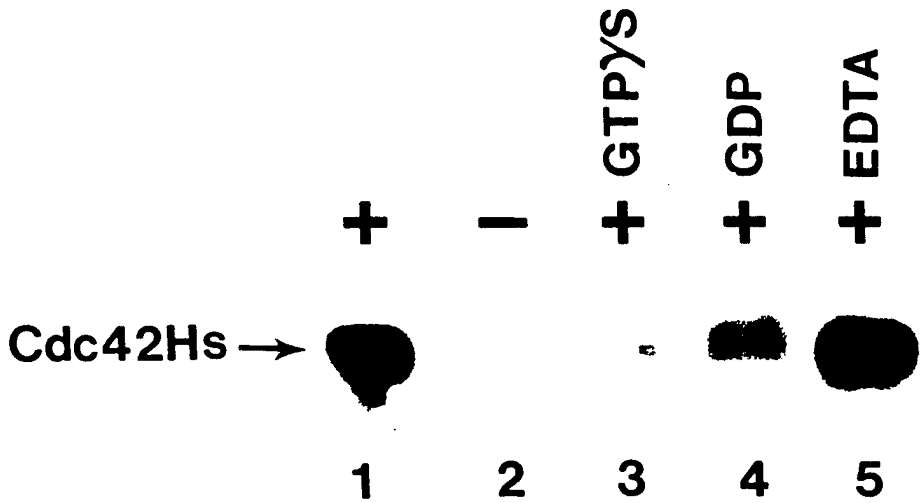
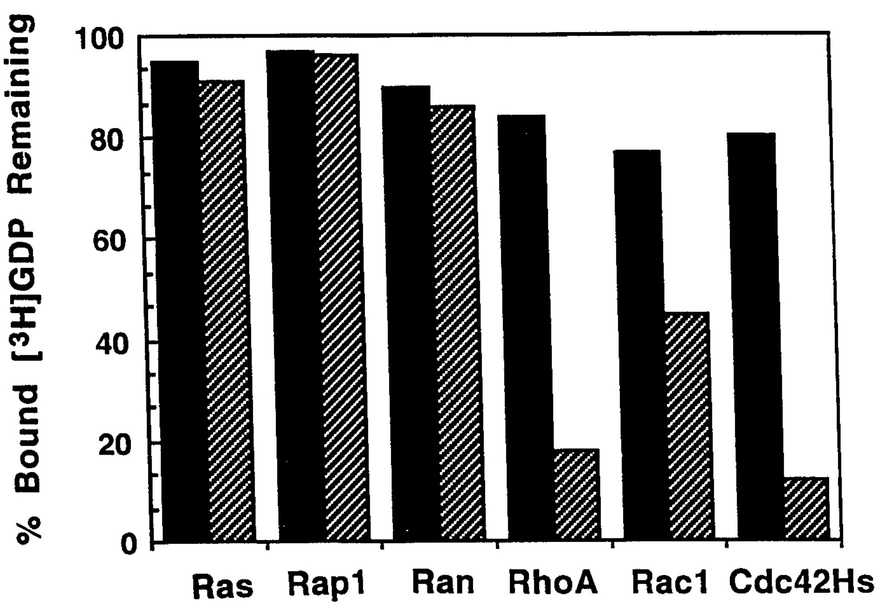



Figure 3



100
80
60
40
20
0
% Bound [3H]GDP Remaining
Ras
Rap1
Ran
RhoA
Rac1
Cdc42Hs


Figure 4

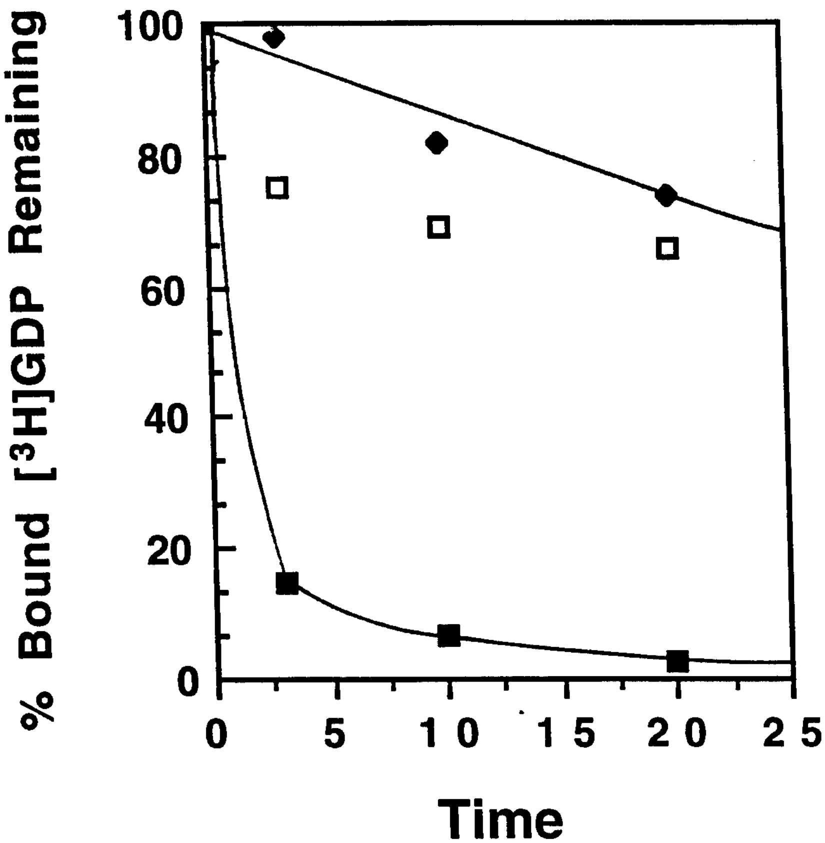



Figure 5